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[GANN, 48, 227~238; October, 1957]

MODIFICATION OF CHICKEN SARCOMA AGENT BY HETEROLOGOUS ANIMAL PASSAGES*

(Plates XVII~XIX)

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Experimental researches on inflammatory viruses are usually performed on those strains which have been successively inoculated in heterologous animals, but in the case of neoplastic virus successive inolculation is done in homologous animals. There has been no reported case of tumor virus being isolated and fixed in heterologous animals. We have been successful in isolating and fixing an agent (HST virus) from the Yoshida tumor by brain passage employing guinea pigs or mice, but it was demonstrated that the agent has no ability to reproduce the Yoshida tumor, being only an inflammatory agent.[1)]

In the present paper we wish to report on our recent studies using the chicken sarcoma agent, which is universally confirmed as a true neoplastic virus. Our experimental results seem to us to strongly support our postulate that the neoplastic viruses, when isolated and fixed by heterologous animal passages, lose their tumor producing potencies but retain their specific antigenicities.

Isolation and Successive Inoculation of Chicken Sarcoma Agent

The Chiba strain chicken sarcoma was used in our study. The method employed in the isolation is in principle identical to that employed in isolating the agent of the Yoshida tumor (HST virus).[2)]

Isolation of the agent by mouse brain passage

This experiment was conducted in our Pathological Department and in another research laboratory located about 100 miles away on different occasions by different researchers. Thus the experiments were done several times under different conditions but the same results were obtained.

Mice which underwent successive brain inoculations usually do not die, but they sometimes become somewhat sensitive. Seven to ten days after the inoculation the mice were sacrificed and their viscera were subjected to histological

* Supported by a Research Grant from the Education Department of Japan.

examination. In the lung viral interalveolar pneumonia, which is well known in viral diseases and neoplastic virus infection, was noted. Hemorrhage and consolidation were seen in one sector and granulative inflammation was relatively strong in the interstices.

In the liver of some cases granulative foci composed mainly of mesenchymal cells were seen and in other cases necrotic or necrobiotic foci of various sizes appeared scatteringly in the parenchyma. In the spleen growth and swelling of the follicles and the proliferation of the reticular cells in the red pulp could be clearly seen. In the brain infiltration of the small round cells into the subarachnoid and proliferation of the subependymal glial cells could be noted.

Isolation and fixation of the agent by guinea pig brain passages

The isolation of the agent of chicken sarcoma is not difficult by mouse brain passage just as we have experienced in the case of HST virus, but the fixation is somewhat difficult. Guinea pigs not only sufficiently compensate for this drawback, but also were convenient in ruling out spontaneous dormant viruses often found in the case of mice. A good number of strains of chicken sarcoma agent we have successfully isolated are preserved in the fixed state in guinea pig brains. The results obtained in the isolation of this agent with the use of guinea pigs have always been identical on several occasions regardless of the difference in data, place, or researcher. The agent was successively inoculated into brains at intervals of 7-10 days, but clinical symptoms hardly appeared in the guinea pigs. The characteristic result found in the experiment with the guinea pigs is that the agent possesses a unique pneumotropic nature. Viral pneumonia was very marked with the consolidation of the parenchyma and with the infiltration of round cells and eosinophilic leukocytes. The impressive finding is that mesenchymal cells and round cells closely converge about the small arteries to form nodules. The findings in the liver are not marked. Groups of mesenchymal cells and small round cells can be seen in the walls of the portal vein and some times localized necrobiosis or necrosis can be noted in the parenchyma.

When placed in 50% glycerin-saline solution the toxicity of this agent is maintained for about 100 days, while in a freezing-dryied state the toxicity is maintained for more than one year.

Isolation of the agent by hamster brain passage

Hamsters are often used in isolating viruses of infectious diseases, but it is reported that they are not very suitable to isolate tumor agents. We used hamsters in isolating the agent of chicken sarcoma, but the fixation was more difficult than in mice. In the lung viral pneumonia resembling that appearing in guinea pigs was seen, but the lesions were slight. The outstanding findings seen in the liver were the proliferation of the small bile duct and mesenchymal cells.

causing a thickening and proliferation of the Glissions sheaths, which in some serious cases produced an annular cirrhosis (Fig. 1).

Successive inoculation of the agent using embryonated chicken eggs.[3]

Brain emulsion of mice inoculated with the agent was placed in the centrifuge. One drop of the supernate thus obtained was inoculated into the chorio-allantois membrane of embryonated eggs and examined 5-7 days later. There was a congestion of the chorio-allantois with diffuse and cloudy thickening and a good number of pock formation was also seen. There was a thickening of the ectodermal epithelium with cornification and desquamation, causing ulcers. Epithelial bodies of various sizes were formed in the subepithelial areas. In the mesoderm there was a proliferation and thickening of the mesenchymal cells with congestion, hemorrhage, edema, etc. There was also an infiltration of small round cells and eosinophilic leukocytes in the perivascular region. The antigenicity of the agent which underwent egg culture is strong and specific, fit for immunization tests. (See the next chapter).

Isolation of the agent from the viscera of animals fed with chicken sarcoma

Bürger,[4] Hayashi,[5] Oshima,[6] and others have reported on the repeated feeding of chicken sarcoma to chickens. Tumor did not develop, but it was demonstrated that a latent infection by Rous virus developed. We isolated and fixed the agent by passing through the mouse brain in the above-mentioned procedure from the lung and liver of chicken fed with chicken sarcoma. This agent caused the same histological changes in the mice as that caused by the agent directly isolated from the sarcoma, and it was proven by cross neutralization tests that this agent and the non-neoplastic chicken sarcoma agent and Rous virus are all of the same species. (See the table).

We have also conducted feeding of chicken sarcoma to mice, rabbits, and hamsters and have confirmed that there takes place an infection and a productive inflammation caused by the agent in each of the animals.

Sero-Immunological Properties of the Isolated Agent of the Chicken Sarcoma

As related above, the agent isolated and fixed by passage through heterogeneous animals was inoculated on chicken and it was confirmed that tumors do not develop. However, we have assumed from our experience on the agent of the Yoshida tumor that they possess a specific antigenicity. Therefore, the following immunization tests were conducted.

Active immunization of chicken

As antigen the supernate of the 10-fold diluted emulsion of the lung of guinea pigs or the chorioallantois of embroyonated eggs which were inoculated res-

pectively with non-neoplastic agent obtained by passage through heterogeneous animals, was used. The supernate was intramuscularly injected in adult chicken at 5–7 days intervals for 9 times to make a total of 44–50cc.

Disturbances developed in the growth of the chicken thus injected and a good number died after much exhaustion. As an adjuvant anhydrous lanoline and liquid paraffin were mixed in the agent and when injected, the amount of the injection was reduced to 16cc. Thus the health of the test animals was disturbed to a lesser degree, which enabled us to fulfil our objectives. The chicken which perished during the immunization were autopsied and studied. In the lungs viral pneumonia was noted and in the liver inflammatory granulation composed of small round cells and mesenchymal cells was seen. These are identical to the histological changes seen in the latent infection of Rous virus.

A total of 49 chickens were used in the experiment. Excluding the 16 chickens which perished during the immunization, chicken sarcoma was transplanted in the breast muscles of 33 chickens two weeks after the last injection of the antigen. No tumors developed in 23 chickens, but in one case a tumor of a forefinger tip size developed after one week. This tumor did not subsequently increase in size but absorbed within two weeks.

According to Oshima,[6] mucous sarcoma locally develops in chicken witn latent infection of Rous virus, when India ink was injected or when a fracture was experimentally set up. Therefore, 8 cases among the above test animals bearing sarcoma immunity were given Indian ink injection or were set up with bone fractures, but we could not observe any tumor development.

25 chickens were used as controls. In accordance to the origin of the agent used in the experiment, the emulsion of the normal mouse brain or the emulsion of the guinea pig lung were injected under the same conditions as in the ordinary experiment and the transplantation of sarcoma was also conducted under similar conditions. All cases developed sarcoma and perished within one month.

In view of the above experiment, it is felt that the agent we isolated by passage through heterogeneous animals should be called non-neoplastic chicken sarcoma agent.

Cross Neutralization Tests

Since 1950 Hamazaki *et al.* have obtained 8 kinds of filtrable agents (Yoshida tumor, MTK tumor, Takeda sarcoma, Hirosaki sarcoma, Usubuchi sarcoma, Brown-Pearce tumor, and Ehrlich ascites cancer) capable of successive inoculation from 16 kinds of transplantable tumors. It was found that embryonated eggs, mice, hamsters and rabbits are susceptible to these agents, but guinea pigs above all are most readily infected by these agents, causing viral pneumonia.

Therefore, it was necessary for us to ascertain the discrimination existing between the non-neoplastic chicken sarcoma agent with which we are dealing in this paper, and the agents of the above mentioned tumors as well as the Rous virus. Cross immunization tests were also conducted using New Castle disease virus as an example of virus for the natural infection of chicken, choriomeningites virus as that for the natural infection of mice, and vaccinia virus as that for rabbits. The homogenate of chicken sarcoma (Chiba strain) was diluted tenfold with normal saline solution and its extract placed in a centrifuge. The supernate thus obtained was used as Rous virus.

For the non-neoplastic chicken sarcoma agent, that agent which was, as previously mentioned, successively inoculated on guinea pig brains was employed.

The Brown-Pearce cancer agent[7] is that which Hamazaki *et al.* isolated and fixed from Brown-Pearce cancer by passage through guinea pig brains. Its properties closely resemble those of other tumor agent we have isolated.

As for New Castle disease virus, we used the Miyatera strain provided by the Livestock Health Laboratory of the Ministry of Agriculture and Forestry.

As for lymphocytic choriomeningitis virus, we used that provided by Dr. Satterwhite of the 406 th Medical General Laboratory.

The glycerized vaccinia virus available on the marked was passed through the testicles of rabbit to increase its virulence. This was used as vaccinia virus.

As previously reported[1], rabbits were immunized by the above viruses and Cross neutralization tests were conducted with their sera.

In determining the results, microscopic examinations were made on the visceral changes of mice inoculated with neutralized agents. Attention must be given to the fact that the titre judging from the histological determination appears generally more markedly than that of the all-or-none (death or surviral)[8] evaluation hitherto employed in determining the virulency of inflammatory viruses.

These results are summarized in Table 1. Cross neutralization between the non-neoplastic chicken sarcoma agent and Rous virus was found to be definitely positive. This phenomenon and the fact that tumor immunity against chicken sarcoma is created when the non-neoplastic chicken sarcoma agent is employed in immunizing chicken as above mentioned, provide proof that both are the same kind of agent.

Cross neutralization with other agents has not been possible. It appears that there is a slight neutralization between choriomeningitis virus and the non-neoplastic chicken sarcoma virus, but since non-neoplastic chicken sarcoma virus and Rous virus could be definitely identified we believe that it does not bear any important gignificance.

Table 1. Immunological Distinction of nn Chicken Sarcoma Agent From Other Agents by Cross-Neutralization

Test Virus	Virus Source	Rabbit Serum	Test Host	Route Inoculated	No. Hosts Inoc. per Virus Dil'n.	Neg. Log ID_{50}
nn Chicken sarcoma agent	mouse brain	normal	mouse	intracereb.	3	>7.0
〃	〃 〃	anti-nn chicken sarcoma agent	〃	〃	〃	2.5
〃	〃 〃	new castle disease	〃	〃	〃	>7.0
New castle disease	CA membrane	anti-new castle disease	〃	〃	〃	4.5
〃	〃 〃	anti-nn chicken sarcoma agent	〃	〃	〃	>7.0
nn Chicken sarcoma agent	mouse brain	anti-nn chicken sarcoma	〃	〃	〃	3.0
〃	〃 〃	anti-LCM	〃	〃	〃	4.8
Lymphocytic choriomeningitis	〃 〃	anti-LCM	〃	〃	〃	3.8
〃	〃 〃	anti-nn chicken sarcoma agent	〃	〃	〃	5.3
nn Chicken sarcoma agent	guinea pig brain	normal	〃	〃	4	7.5
〃	〃 〃 〃	anti-nn chicken sarcoma agent	〃	〃	〃	3.7
〃	〃 〃 〃	anti HST. V	〃	〃	〃	7.0
Yoshida tumor agent (HST. V)	〃 〃 〃	anti HST. V	〃	〃	〃	4.0
〃	〃 〃 〃	anti-nn chicken sarcoma agent	〃	〃	〃	6.5
nn Chicken sarcoma agent	guinea pig brain	normal	〃	〃	〃	7.7
〃	〃 〃 〃	anti-nn chicken sarcoma agent	〃	〃	〃	4.2
〃	〃 〃 〃	Rous chicken sarcoma virus	〃	〃	〃	4.0
Rous chicken sarcoma virus	〃 〃 〃	normal	〃	〃	〃	7.0
〃	〃 〃 〃	anti-Rous chicken sarcoma virus	〃	〃	〃	4.0
〃	〃 〃 〃	anti-nn chicken sarcoma agent	〃	〃	〃	4.5
nn Chicken sarcoma agent	mouse brain	normal	〃	〃	〃	7.2
〃	〃 〃	anti-nn chicken sarcoma agent	〃	〃	〃	4.5
〃	〃 〃	anti-vaccinia	〃	〃	〃	7.5
Vaccinia	rabbit testicle	anti-vaccinia	〃	〃	〃	2.8
〃	〃 〃	anti-nn chicken sarcoma agent	〃	〃	〃	5.7
nn Chicken sarcoma agent	guinea pig brain	normal	〃	〃	3	7.0
〃	〃 〃 〃	anti-nn chicken sarcoma agent	〃	〃	〃	4.0
〃	〃 〃 〃	anti Brown-Pearce agent	〃	〃	〃	6.6
Brown-Pearce agent	〃 〃 〃	anti Brown-Pearce agent	〃	〃	〃	3.5
〃	〃 〃 〃	anti-nn chicken sarcoma agent	〃	〃	〃	7.0

Return Inoculation of Non-Neoplastic Chicken Sarcoma Agent Into Chicks

From the immuno-serological studies reported in the provious chapters, it has been verified that this agent is a variety of the Rous virus, which has, like the HST virus, lost its neoplastic ability but yet maintains only its specific antigenicity, and is an agent different in kind from the other inflammatory viruses. Various constitutional differences between this agent and Rous virus (Chicken sarcoma virus) can be considered, but perhaps those with the greatest probability lie in the relation with the chicken soma cell protein. It was thus felt that when a return inoculation of the non-neoplastic chicken sarcoma agent was done from the guidea pig to the chicken, followed by successive inoculations, neoplastic ability may be restored with the possible annexing of chicken tissue factor. Therefore, a return inoculation of this agent on chicks which are most sensitive to chicken sarcoma agent was attempted.

The agent was injected in the abdominal cavity of chicks and inoculation was done at intervals of two weeks. The liver emulsion supernate of these chicks was injected in chicks of the succeeding generations.

During the early stage of the successive inoculations hemorrhagic necrotic inflammation[9] appeared in the intestines of the relatively young chicks as in the case of infection by chicken sarcoma virus, while in the liver focal necrosis was noted. In many cases the chicks died of exhaustion. However, in 25% of the cases primary foci of chicken sarcoma variation, as Borges and Duran-Reynals have reported[10], and their metastatic tumors, that is sarcoid foci closely resembling a typical granuloma, were seen in the liver, lung, intestines, and spleen. In other cases granulative inflammation resembling lesions caused by the infection of chicken sarcoma virus was seen in the liver, spleen, and kindney, while in the lung viral interalveolar pneumonia was noted.

Small pieces of liver, lung and spleen of chicks having such lesions were transplanted in the muscles of 2-3 months old chickens. 13 cases out of the 24 developed fibromyxoma and among the four of this group tumor growth was especially outstanding. The tumor cells appeared fibroblastic or fibrocytic and ran in bundles in an interlacing manner with scattered mitosis, while the polynuclear giant cells were seen in strata. Mucus production of the tumor was demonstrated by mucicarmin and Schiff reaction. In one of other tumors a picture resembling round cell sarcoma was seen. Three cases out of the four with outstanding tumor growth were capable of successive transmissions. Two cases out of the 24 developed sarcoid-like lesions in the liver which are considered to be equivalent to fibromyxoma metastasis by Borges and Duran-Reynals

developing from visceral transplantation of chicken sarcoma bearing pigeons. These sarcoid granulation could be successively inoculated from liver to liver.

Six adult chicken were immunized using the agent which underwent passage through these chicks. Five cases of these six chickens developed complete tumor immunity, and one case died during the immunization. In the viscera inflamatory granulation resembling the sarcoid granulation, as previously mentioned, developed. Though the cell free suspension of this agent was inoculated in chicken, tumor formation could not be demonstrated.

As indicated in these tests, when return inoculation of non-neoplastic chicken sarcoma agent into chicks is performed, transplantable fibromyxoma or a variation of chicken sarcoma appeared[11]. It can be considered that this is an indication that its neoplastic ability has been restored to a certain extent.

According to the kind of animals infected with tumor virus, neoplastic ability of tumor virus was in this manner lost and was later restored by successive transplantations into the native hosts. This phenomenon has also been noted with rabbit fibroma, cottontail rabbit papilloma, and Fujinami chicken sarcoma. It can be assumed that in the so-called masked infection caused by these tumor agents there is an indication that the second factor originating from the host cells is absent as in the case of non-neoplastic chicken sarcoma agent.

Feeding Tests of Chickens With Chicken Sarcoma

Bürger[4] has conducted extensive feeding tests of this kind in the past, but has been unsuccessful in reproducing tumor used in the feeding tests. However, these chickens which underwent feeding tests began to show a strong resistance against the transplantation of chicken sarcoma. According to Oshima,[6] the chickens themselves which were fed with the sarcoma did not produce any tumors, but a latent infection of virus was created. Evidently due to this infection, when the liver and lung tissues of these chichens were transplanted in normal chicken, sarcoma developed. We have also made extensive feeding tests with chicken sarcoma and like other researchers failed to produce any tumors. When mouse brain passage was done with the liver and lung tissues of chicken fed with chicken sarcoma, a certain filtrable agent was isolated, which was identified by cross neutralization with the non-neoplastic chicken sarcoma virus as above mentioned.

Feeding tests of other animals (mouse, rabbit, and hamster) with chicken sarcoma have also been done in our laboratory and in all cases viral granulative inflammation was noted in the viscera. We were able to isolate the non-neoplastic chicken sarcoma agent from the viscera of these animals, but tumors were not formed.

Discussion

It is well known that a latent infection may often exist in neoplastic viruses. It is assumed that when they are provoked by a proper mechanical or chemical stimulation, they may be able to induce cancers. Chicken sarcoma virus (Rous) is one of the viruses which most evidently supports this assumption. Rous virus which is the neoplastic agent extracted directly from chicken sarcoma could generally satisfy Koch's postulates. As pure culture cannot be made with virus as in the case of bacteria, isolation and fixation are done by passage through heteaogeneous animals. This method had been neglected with tumor viruses. Hamazaki *et al.* have in the past isolated the agent of the Yoshida tumor and have recently isolated the agents of chicken sarcoma, rabbit myxoma, and Brown-Pearce cancer in the same manner. However, when isolated and fixed in heterogeneous animals, the agent generally loses its original pathogenicity against homologous animals, but only maintains its antigenecity.[11)12)] This has been known through classical studies on rabies virus and vaccinia virus. From our experiments we have discovered that this principle can also be applied to neoplastic viruses. In this sense the pure culture of bacteria and the animal passage of viruses cannot be considered to have the same value.

Even with Rous virus, sarcoma cannot be produced unconditionally. In case of natural infection, that is oral or fetal infection, sarcoma is not produced but a latent infection is created. However, if an artificial stimulation is provided, sarcoma is produced. No matter how great an amount of non-neoplastic chicken sarcoma virus isolated by Hamazaki may be inoculated in chickens, sarcoma is not produced and even when the infected animal is provided a stimulation, sarcoma cannot be produced which is contrary to the case of the original Rous virus. However, we were able to confirm immuno-serologically that Hamazaki agent belongs to the one of those similar to the Rous virus. It is said that an experimental immunization against markedly malignant tumors such as chicken sarcoma is very difficult to produce, but such immunization could be relatively easily produced by employing non-neoplastic chicken sarcoma agent as antigen.

Where is the essential difference between the Original Rous virus and the non-neoplastic chicken sarcoma agent? As the non-neoplastic chicken sarcoma agent has undergone frequent passage through heterologous animals, it proliferates in protein milieu unlike that of the original host and variation must have developed. Further studies should be made to determine whether these are true mutations or not. For this purpose, successive return inoculations were made in chicks with the non-neoplastic chicken sarcoma agent and pieces of their lung and liver tissues were transplanted in the thoracic muscles of adult chickens. Thus we

were able to induce transplantable tumors capable of producing mucus. As the malignancy of this tumor is mild, we cannot say that the non-neoplastic chicken sarcoma agent transformed itself back to the original Rous virus, but it is felt that there is sufficient evidence to support this possibility.

Conclusion

1. A filtrable agent could be isolated from chicken sarcoma by passage through the brains of guinea pigs, mice, and hamsters regardless of place or time. The agent fixed in guinea pigs is the most stable and toxic of those fixed in those animals.

2. When this agent was inoculated in chickens, they developed granulative inflammation which never accompanies any tumor formation. When it was successively inoculated in chicks, sarcoid-like foci developed with a pathological picture resembling that reported by Borges and Duran-Reynals, but no sarcoma was formed.

3. The chickens immunized with the agent refused the growth of transplanted chicken sarcoma with strong immunity.

4. In a cross neutralization test between this agent and the chicken sarcoma virus (Rous) both agents could be perfectly identified. The cross neutralization of this agent with the HST virus, New Castle disease virus, lymphocytic choriomeningitis virus and vaccinia virus was negative.

5. When the liver and lung tissues of chicks which underwent successive inoculations by this agent were transplanted to adult chickens, transplantable fibrosarcoma and fibromyxoma were produced.

6. When chickens, mice, hamsters, and rabbits were fed with chicken sarcoma, granulative inflammation developed in the viscera. We were able to isolate from the viscera of the fed chickens an agent with properties identical to those of the non-neoplastic chicken sarcoma agent.

7. We propose to call this agent the "NN chicken sarcoma agent".

Explanation of Figures (Plates XVII~XIX)

Fig. 1. Hamster No. 87, 2nd generation, in the isolation experiment of the non-neoplastic chicken sarcoma agent from chicken sarcoma by the successive inoculations in hamsters.

Glisson's sheath hypertrophied with augumentation of bile ducts and infiltration of round cells, resulting in pseudo-acini.

Fig. 2. Mouse No. 50/D, 6th generation, in the isolation experiment of the non-neoplastic chicken sarcoma agent from the liver of the chicken fed with chicken sarcoma.

Degeneration of the hepatic cells with inflammatory granulation consisting of proliferation of the stellate cells, mesenchymal cells and a few multinucleated giant cells as well as infiltration of monocytes, lymphocytes and eosinophilic leucocytes.

Fig. 3. Mouse No. 3, the 2nd generation, in the isolation experiment of the non-neoplastic chicken sarcoma agent from the liver of the chicken fed with chicken sarcoma.

In the hepatic interstices centering around the walls of portal vessels as well as bile ducts, proliferation of the mesenchymal cells and infiltration of round cells are seen.

In the parenchyma degeneration of the hepatic cells can be observed, accompanied by infiltration of monocytes and proliferation of stellate cells.

Fig. 4. Chicken No. 6/C, the 3rd immunization test of chicken with cell-free brain-emulsion, originating from guinea pigs infected with non-neoplastic chicken sarcoma agent.

Between the myocardial fibers are seen diffused or localized infiltration of round cells and certain granulomatous nodules resembling rheumatic nodes.

Fig. 5. Chick No. 268/A, the 29th generation of successive inoculations with non-neoplastic chicken sarcoma agent.

Return inoculation into the chicks of the non-neoplastic chicken sarcoma agent obtained from the guinea pigs successively inoculated with the chicken sarcoma virus.

Semi-translucent, granuloma-like lesions (S) occupy the major part of the lung and the remaining normal part appears rather emphysematous. (N)

Fig. 6. Chick No. 64/A2, the 28th generation of the above. The liver showing many greyish-white, round nodes, firm in consistence and projecting slightly. (S) Microscopically the nodes revealed a sarcoid-like lesion.

Fig. 7. Chick No. 16/B, the 16th generation, experimental procedure as above.

B microscopic diagram of a sarcoid-like lesion of the lung. A large necrotic mass surrounded by proliferated mesenchymal cells and multinucleated giant cells, accompanying a round cell wall.

Fig. 8. Chick No. 73/A1, the 30th generation, experimental procedure as above.

A fairly large number of multinucleated giant cells and mesenchymal cells are seen surrounding the necrotic mass of hepatic parenchyma, with slight infiltration of both monocytes and eosinoyhilic leucocytes.

Fig. 9. The breast tumor of a chicken (No. 102) induced by transplanting a piece of the liver of chick inoculated with non-neoplastic chicken sarcoma agent.

The tumor cells contain a relatively small amount of cytoplasm, while their nuclei contain a rather large amount of chromatin showing many mitosis.

Fig. 10. Hamster No. 68 of feeding tests with chicken sarcoma: Due to the proliferation of the septal cells and infiltration of round cells the alveolar septum has become hyperplastic and alveolar spaces are either narrowed or plugged.

References

1) Hamazaki, Y., *et al.*; Studies on the HST (Hamazaki) Virus isolated from Yoshida Tumor. Cancer Res. 13: 119-124. 1953.

2) Hamazaki, Y., *et al.*: Experimental induction of cancer by feeding of transplantable tumors. No. 1. Proc. of Japan Acad. 31: 480-485. No. 2, ditto, 486-491. No. 3, ditto, 721-726, No. 4, ditto, 727-732. 1955.

3) Hamazaki, Y., *et al.*; On the inoculalion of the HST (Hamazaki) Virus isolated from Yoshida tumor on embryonated eggs. Acta medica Okayama. 10: 62-69. 1956.

4) Bürger, M.; Ueber das Hühnersarkom. Ztschr. für Krebsforsch. 14: 526-542. 1914.

5) Hayashi, N.; On the chicken tumor. Trans. Soc. Path. Jap. 4: 255-259 1929.

6) Oshima, F.; Pathology of chicken sarcoma. Trans. Soc. Path. Jap. 40: 13-31. 1951.

7) Hamazaki, Y., *et al.*; On the non-neoplastic viruses isolated from viral tumors. Gann. 45: 379-381. 1954.
8) Luria, S. E.; General virology. London P. 25. 1953.
9) Duran-Reynals, F.; Neoplastic infection and cancer. Amer. J. of Med. 8: 490-511. 1950.
10) Borges P. R. J. & Duran-Reynalds, T.; On the induction of malignant tumors by a chicken sarcoma virus to ducks. Cancer Res. 12: 55-58. 1952.
11) Duran-Reynals, T.; In "Physiopathology of Cancer" (Humburger *et al*). A. Hoeber-Happer Book. P. 298. 1953.
12) Shope, R. E.; A change in rabbit fibroma virus suggesting mutation. J. Exp. Med. 63: 173-184. 1936.
13) Andrewes, C. H.; Viruses in relation to the aetiology of tumors. Lancet. 227: 63-69. 1934.

要　旨

異種動物通過による家鶏肉腫病毒の変化

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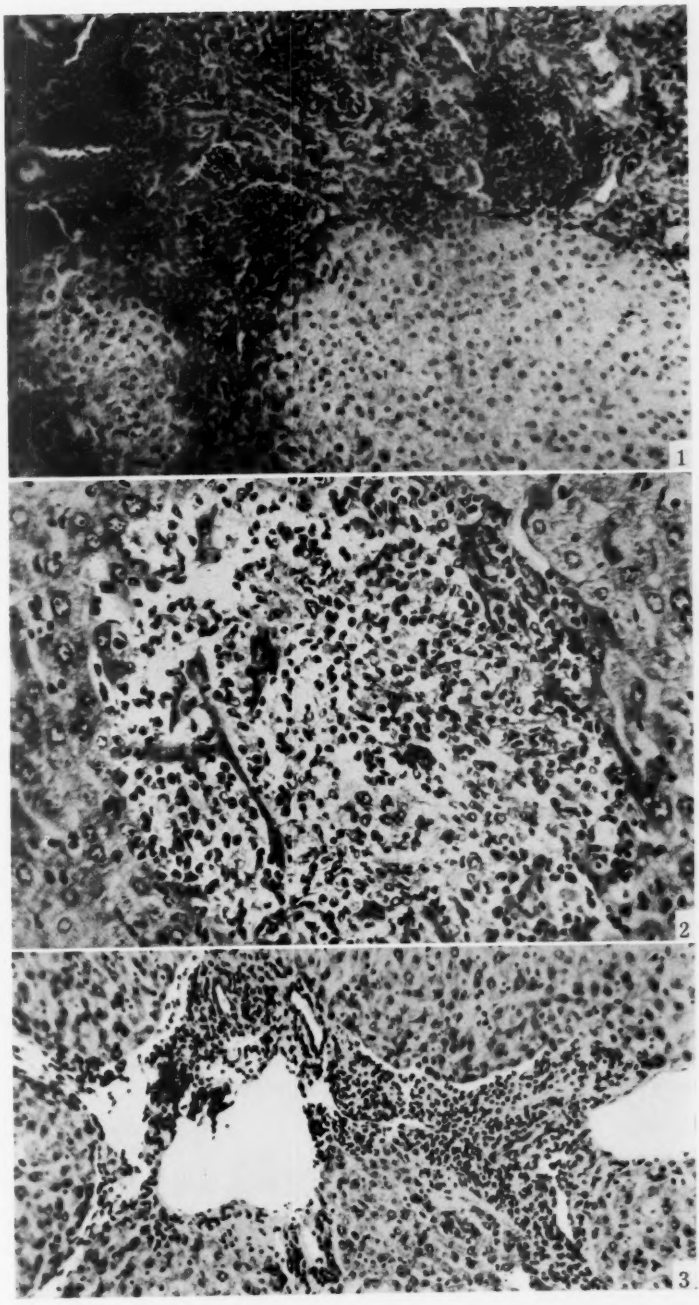
著者等は腫瘍病毒を異種動物脳を通過させて分離する方法を広汎に行い「一般に発癌性因子は異種動物を通過させて分離固定するときはその発癌性を失うが，特殊抗原性はよく保有するものである」ことを提唱した。その最も重要な論拠をなしたのがこの実験である。すなわち千葉系家鶏肉腫ヴィールスを海猿，マウスあるいはハムスター，に異代移植すると宿主動物に肉芽性炎症を起す戸過性病毒が常に得られる。この病毒は交叉中和実験によってラウス・ヴィルスと同種のものであり，ニューキャッスル病，リンパ細胞性脈絡脳炎，吉田腫瘍病毒 (HST. V.) 及び，牛痘ヴィルスとは関係のないことを証明した。

この病毒は成鶏に接種しても腫瘍を生じないが，内臓に肉芽性炎症を起し，家鶏肉腫に対して強い免疫が現れる。これに反しこの病毒を雛に異代接種したのち，雛の肝片を成鶏に移植するときは可移植性の線維粘液腫を得た。これ等の成績から判断すると，家鶏肉腫ヴィルスを異種動物通過によって分離するとき発癌性を失うことは家鶏体細胞に由来する第2因子を失うためと考えられる。従ってこれを雛に継代すると再び腫瘍形成能が得られるのであろう。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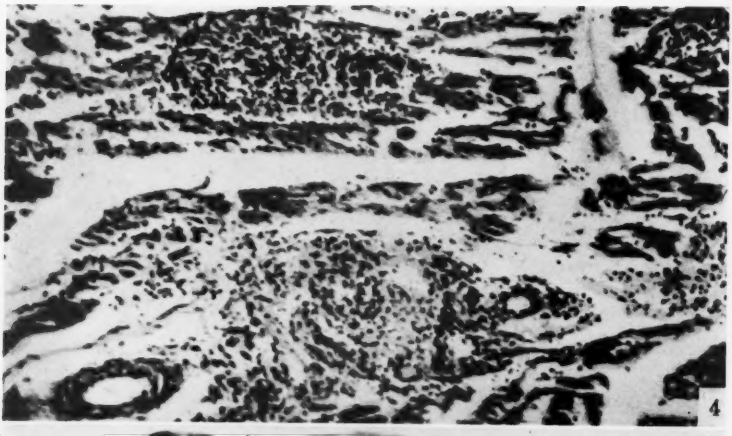
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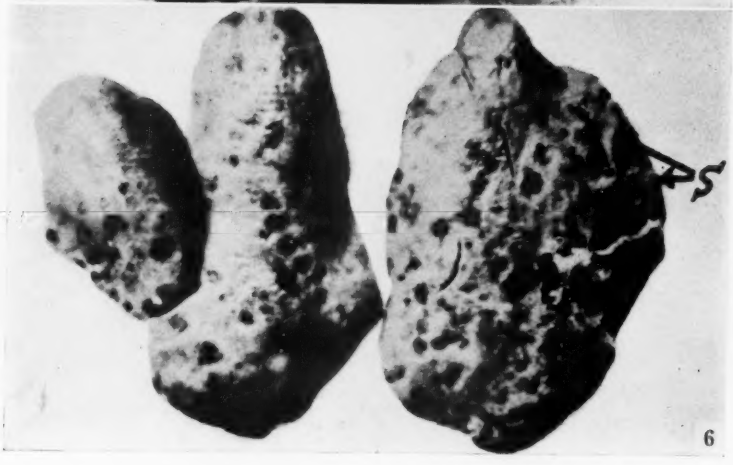

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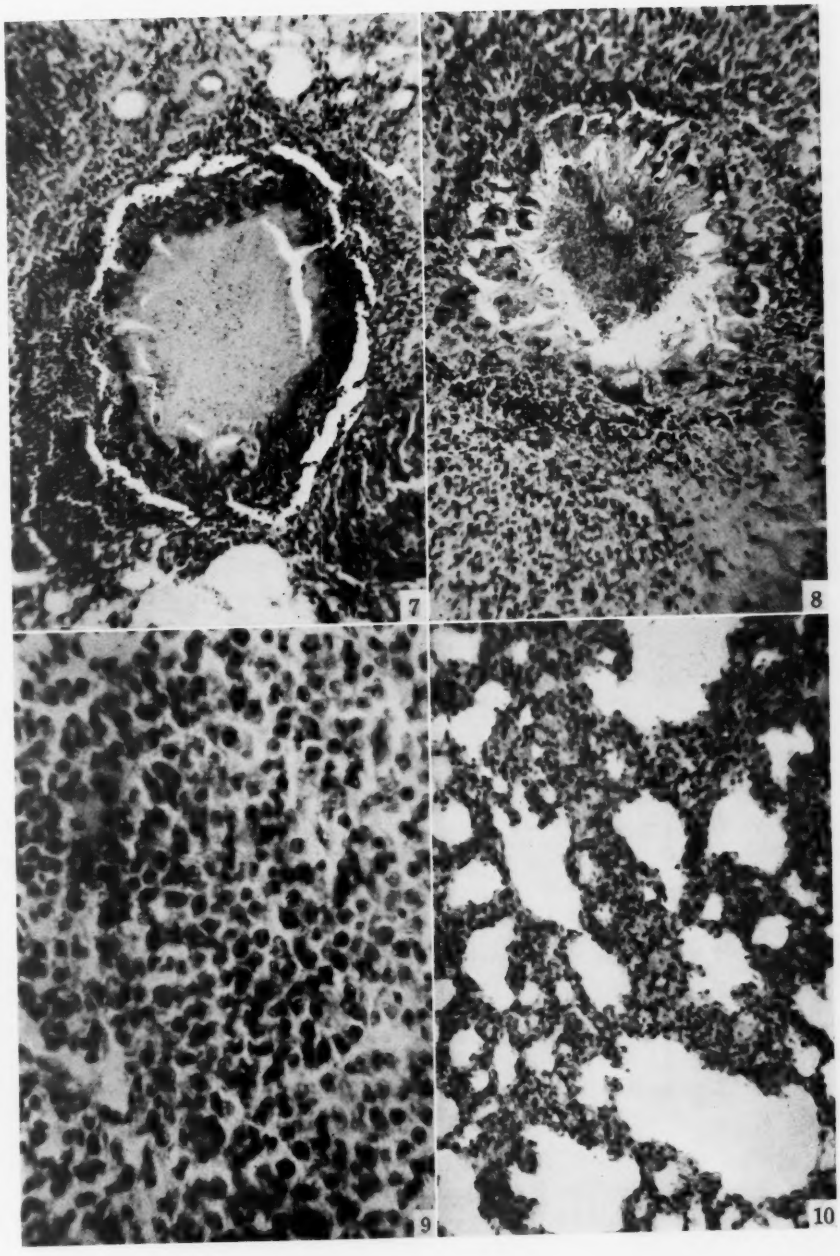
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[GANN 48, 239~242; October, 1957]

STUDIES ON THE MECHANISM OF TOXOHORMONE FUNCTION

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Recently, Greenfield and Price (1, 2) have shown that the tumor bearing animals had a lowered amount of storage iron in the liver and a decreased uptake of Fe^{59} into liver ferritin and liver catalase.

It is well known that the toxohormone prepared from malignant tumors by Nakahara and Fukuoka (3, 4) has an activity of hepatic catalase depression when it is injected into mice, and this toxohormone effect was counteracted by ferric chloride injected (5).

In view of these facts it may be of much interest to study the relation between catalase reducing effect of toxohormone and liver iron metabolism.

In this investigation, storage iron and catalase of the toxohormone treated mice were determined and a parallel depression between storage iron and catalase activity was observed. In agreement with the results of Nakahara and Fukuoka, it was also demonstrated that toxohormone has no inhibitory effect on the crystalline beef liver catalase *in vitro*.

Experimental Material and Methods

Toxohormone: Toxohormone was prepared from surgically removed human gastric cancer tissues* by the method of Ono *et al.* (6).

Crystalline catalase: Crystalline catalase was prepared from beef liver by the method of Sumner and Sarkar (7).

Methods: 50 mg of toxohormone Fraction III was dissolved in the minimum amount of dilute hydrochloric acid and adjusted to pH 6 by alkali and injected intraperitoneally into mice.

Liver catalase activity was assayed as follows: The mice were killed by exsanguination and a piece of liver removed weighing accurately 0.1 g. in torsion balance was homogenized with 5 ml of 0.01 M phosphate buffer of pH 7.0 in a glass homogenizer, and centrifuged at 3000 r.p.m. at 0°C for 5 min. 0.2 ml of the extract thus obtained was diluted with 50 ml of 0.01 M phosphate buffer of pH 7.0, and its catalase activity was measured by the permanganate titration method of Bonnichsen, Chance and Theorell (8). Blowout times of 60, 120 and 180 seconds were employed. The results were expressed exclussively with k according to the

* These materials were kindly supplied by Dr. K. Shimizu of St. Maria Hospital in Himeji.

following equation (uncorrected to 0°C).

$$k = \frac{\ln(x_0/x)}{t} \quad \text{sec}^{-1}$$

x_0 : substrate concentration at time 0
x : substrate concentration at time t
t : time

Ferritin Fe was determined by the method of M. Yonekawa and K. Konno (9), using 0.5 g of fresh liver.

Results

I. Effect of toxohormone on the liver catalase and the liver iron metabolism.

The toxohormone was injected into mice intraperitoneally, and liver catalase activity and ferritin Fe were determined after 20 hours.

The liver catalase activities of normal and toxohormone treated mice are shown in Fig. 1.

Table 1 shows the non-hemin iron contents in the normal liver. PI, PII, PIII

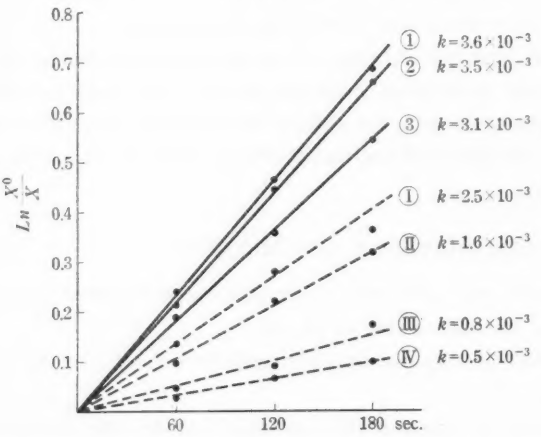


Fig. 1. Liver Catalase Activity of Normal and Toxohormone treated Mice.

Solid Line: Normal mice
Dotted Line: Toxohormone treated mice

Table 1. Non-hemin Iron of normal mice

Exp. No.	PI*	PII*	PIII*	SIII*
4	52	85	10	<1
5	56	94	—	—
6	47	104	—	—
7	—	99	—	—
8	39	70	7	<1
mean value	44	90	9	<1

* γ of Fe/g of fresh liver

Table 2. Non-hemin Iron of toxohormone treated mice

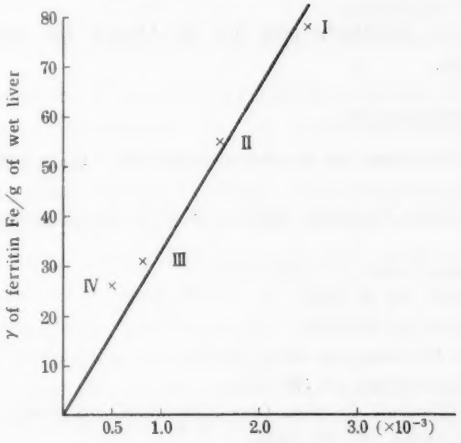
Exp. No.	PI*	PII*	PIII*	SIII*	k
I	—	78	—	—	2.5×10^{-3}
II	35	55	11	<1	1.6×10^{-3}
III	46	31	13	<1	0.8×10^{-3}
IV	27	26	3	<1	0.5×10^{-3}
V	49	61	20	<1	—
mean value	39	50	12	<1	—

* γ of Fe/g of fresh liver

and SIII represent hemosiderin, ferritin, nucleoprotein bound iron, and free iron respectively.

Table 2 shows the non-hemin iron contents in the liver of toxohormone treated mice. Catalase activity of each liver (k) was also placed in the Table for reference.

Fig. 2. Parallelism between ferritin iron and liver Catalase.



I—IV: Experimental Numbers in Table II.

As compared with Table 1, only PII fraction (ferritin iron) of the Table 2 showed a remarkable depression.

Furthermore the depression of ferritin iron was in parallel with the reduction of catalase activity in the same liver. This relation can be cleary seen in Fig. 2.

II. Effect in vitro of toxohormone on the crystalline liver catalase.

Crystalline beef liver catalase was dissolved with 0.01 M phosphate buffer of pH 7.0, and $2.5\times10^{-3}\mu$M catalase solution was obtained (estimated by optical density at Soret band 405 mμ). 96 ml of this solution was divided into two flasks. 5 mg of toxohormone (Fraction III) was dissolved into the one flask. 2 ml of 0.25 N-H_2O_2 was added to each flask, and catalase activity was determined immediately. The toxohormone added *in vitro* does not exhibit any inhibitory action on crystalline beef liver catalase. The catalase activities of two flasks had the same k value (3.0×10^{-3}).

Discussion

Recently Sugimura (11) has reported that the ferritin iron content of tumor bearing animals showed no remarkable change compared with normal.

Theorell's observation (10), however, seems to suggest that the first response in the liver iron metabolism of cancer host may occur in the ferritin iron. The present result, together with the reports of Greenfield and Price (1, 2) referred to in the preface of this paper have suggested that the ferritin iron and liver catalase iron might be in a closely equilibrated state.

No explanation for this discrepancy is available at present.

Summary

Toxohormone prepared from human gastric cancer induced a paralled depression of ferritin iron and liver catalase of mice, and showed no effect on the crystalline beef liver catalase *in vitro*.

Acknowledgment

The author is indebted to Prof. S. Mizuhara and Mr. K. Okuda for their generous advice throughout this work.

References

1) Greenfield, R. E., and Price, V.E., Cited from the lectures of Greenstein, J.P., in Japan, 52 (1956)
2) Greenfield, R.E., and Price, V.E., Cancer Research, Abstract of 47 th Annual Meeting, Vol. II, 111 (1956)
3) Nakahara, W., and Fukuoka, F., Japan Med. J., 1, 271 (1948)
4) Nakahara, W., and Fukuoka, F., Gann, 40, 45 (1949)
5) Fukuoka, F., and Nakahara, W., Gann, 42, 55 (1951)
6) Ono, T., Sugimura, T., and Umeda, M., Gann, 46, 617 (1955)
7) Sarkar, N.K., and Sumner, J. B., Enzymologia 14, 280 (1951)
8) Bonnichsen, R. K., Chance, B., and Theorell, H., Acta Chem. Scand., 1, 685 (1949)
9) Yonekawa, M., and Konno K., J. Biochem., 40, 377 (1953)
10) Theorell, H., Benzak, N., Bonnichsen, R. K., Paul, K. G., and Akeson, A., Acta Chem. Scand., 5, 443 (1951)
11) Sugimura, T., Gann, 48, 73 (1957)

要　旨

Toxohormone の作用機構に関する研究

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人の胃癌組織より精製した toxohormone を廿日鼠に投与し，肝臓の non-hemin 鉄を測定したところ，ferritin 鉄劃分のみの減少がみられ，かつその減少は catalase の減少と平行していることを見出した。またこの toxohormone は結晶牛肝カタラーゼには何等抑制作用を示さなかった。

(文部省科学研究費による)

[GANN, 48, 243~248; October, 1957]

SOME CHEMICAL CHANGES IN THE LIVER OF RATS FED p-DIMETHYLAMINOAZOBENZENE. I.

GENJIRO DOI

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Studies of the toxohormone effect on the iron metabolism by Hoshijima[1] is now being conductuted. In this connection, papers of this series will, it is hoped, offer some information on the iron metabolism of rat liver in the course of carcinogenesis caused by p-dimethylaminoazobenzene (DAB).

Nakahara *et al.*[2] and Yanagizawa[3] have independently reported that the liver catalase activity of rats fed DAB is lowered long before the appearence of hepatoma. This DAB effect, however, is quite puzzling. Nakahara *et al.*[4] have reported that DAB can directly inhibit the liver catalase *in vitro*, while Miyaji *et al.*[5] obtained a contrary result using liver homogenate.

It should also be recalled that ascorbic acid has an intimate relation with the iron metabolism[6] and most of the flavoproteins contain some metal ions (Fe, Cu, Mo) as the integral part of enzymes.[7] This communication will show the effect of DAB on the crystalline catalase and the changes of contents of catalase, Fe, Cu, ascorbic acid, and riboflavin in the rat liver after DAB feeding for about 30 days.

Material and Methods

Crystalline beef liver catalase was prepared by the method of Sarkar and Sumner.[8]

The activity of crystalline catalase was assayed by the ultraviolet spectroscopic method of Chance and Herbert.[9][11]

Catalase activity of the liver homogenate was assayed by the modified method of Bonnichsen *et al.*[10][11]

Non-hemin iron of the liver was determined by the method of Yonekawa and Konno.[11]

Copper content of liver was estimated by the wet ashing method of Eden and Green.[12] Total ascorbic acid of the liver was determined by the method of Roe *et al.*[13]

Total flavin was determined by the fluorophotometric method of Yagi.[14] Rats were fed on a diet containing DAB (0.6 g of DAB in 20 ml of olive oil was mixed with one kg of rice) for about 30 days. Then the rats were killed by exsanguination and the determinations were carried out with the liver removed.

Results

I. Effect of DAB on the Crystalline Beef Liver Catalase.

Crystalline beef liver catalase was dissolved in 0.01 M phosphate buffer of pH 7.0 and its concentration was estimated to be 7.4×10^{-2} M by measuring the optical density at 405 mμ. 0.03 ml of this catalase solution was rapidly stirred into 3 ml of the H_2O_2 solution (0.01 N) which was placed in a Beckmann cuvette. The changes of concentration of H_2O_2 were pursued at every 60 second interval by measuring optical density at 240 mμ, using Beckman spectrophotometer. DAB added was 5γ per ml of H_2O_2 solution. In the final solution, DAB content was about five thousand times more than catalase in the molecular basis. Optical density at 240 mμ of the DAB containing solution gave a higher value than the solution containing no DAB. But the difference between two solutions above described was constant in the range of 220-260 mμ. So the correction of the higher values observed by added DAB was easily attained.

The catalase activity is expressed by k of the following equation:

$$k=\frac{Ln\frac{x_0}{x}}{t}$$

Where x_0 is the substrate concentration at zero time, and x is the substrate concentration at time t. The temperature of the incubation mixture was 27°C. The results, as shown in Fig. 1, indicate that DAB does not inhibit the crystalline beef liver catalase *in vitro* even in such a high concentration.

Fig. 1 DAB Effect on the Crystalline Beef Liver Catalase

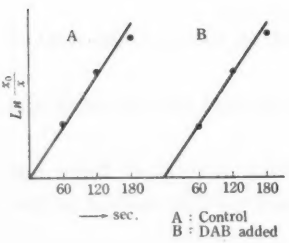
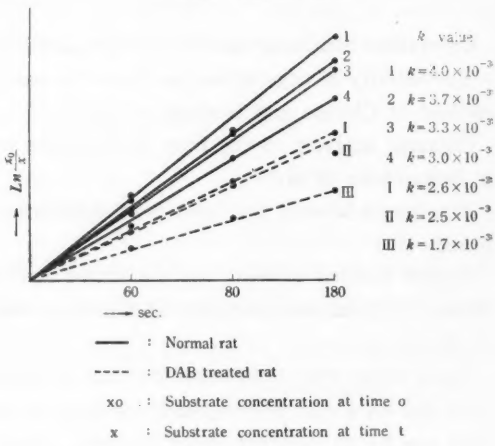


Fig. 2 Liver Catalase Activity of Normal and DAB Treated Rats



II. Liver Catalase Activities of Normal Rats and Those Fed DAB.

As shown in Fig. 2, the catalase activity of the liver was decreased in rats fed DAB.

III. Iron Contents in the Liver of Normal Rats and Those Fed DAB.

Table 1 shows the iron content of the normal rat liver. In this table PI, PII, PIII, and SIII have been proposed to represent hemosiderin, ferritin, nucleoprotein bound, and free iron respectively.[11] Table 2 shows the iron content of the liver of rats fed DAB. From these tables, it is clearly seen that the liver hemosiderin iron (PI) of rats fed DAB is considerably higher than normal, and the ferritin iron (PII) is slightly higher than normal. A different degree of susceptibility toward the carcinogenic agent has been often observed from strain to strain. Similar situation was also seen in the reduction of liver catalase of rats fed DAB. A parallel relation, however, was observed between catalase depression and the increases of hemosiderin and ferritin.

Table 1. The Iron Content in the Normal Rat Liver (expressed by γ/g of wet liver)

Exp. No.	PI	PII	PIII	SIII
1	26	115	4.6	1.8
2	52	140	10.0	1.5
3	34	66	3.9	1.3

Table 2. The Iron Content in the DAB Fed Rat Liver (expressed by γ/g of wet liver)

Exp. No.	PI	PII	PIII	SIII	Catalase activity (k)
4	188	138	11.0	0.76	2.6×10^{-3}
6	116	134	6.6	1.5	2.5×10^{-3}
7	212	260	7.6	1.5	1.7×10^{-3}
8	55	75	4.6	1.2	3.7×10^{-3}

IV. Copper Contents in the Liver of Normal Rats and Those Fed DAB.

Copper is known to be essential for the utilization of iron in the body.[15] As shown in Table 3, copper content in the liver of rats fed DAB was lowered compared with the normal. The reduction of copper content was paralled with the catalase depression in the same liver.

Table 3. Liver Copper Contents of Normal and DAB Fed Rats (expressed by γ/g of wet liver)

Normal rat		DAB fed rat		
Exp. No.	Copper content	Exp. No.	Copper content	Catalase activity (k)
1	19.5	7	11.1	2.6×10^{-3}
3	25.0	8	10.5	2.5×10^{-3}
5	17.4	9	8.4	1.7×10^{-3}
6	26.5	11	18.8	3.7×10^{-3}

V. Ascorbic Acid Contents in the Liver of Normal Rats and Those Fed DAB.

Tables 4 and 5 show the total ascorbic acid contents in the liver of normal rats and those fed DAB respectively. Ascorbic acid was increased in DAB fed rats. Only in this case a parallelism is not observed between catalase depression and increase of ascorbic acid.

VI. Total Riboflavin Contents in the Liver of Normal Rats and Those Fed DAB.

Table 4. Total Ascorbic Acid in Normal Rat Liver

Exp. No.	Total ascorbic acid (γ/g of wet weight)
9	230
11	174
12	224
14	248

Table 5. Total Ascorbic Acid in the Liver of Rats Fed DAB (γ/g of wet weight)

Exp. No.	Total ascorbic acid	Catalase activity (k)
15	335	2.6×10^{-3}
16	347	2.5×10^{-3}
18	245	1.7×10^{-3}
19	360	3.7×10^{-3}

Table 6. Total Flavin Contents of Normal and DAB Fed Rat Liver (expressed by γ/g of wet liver)

Normal rat		DAB fed rat		
Exp. No.	Total flavin content	Exp. No.	Total flavin content	Catalase activity (k)
1	13.5	4	7.6	1.7×10^{-3}
2	11.6	5	6.9	2.6×10^{-3}
3	12.1	7	8.5	2.6×10^{-3}
		8	10.4	3.7×10^{-3}

Table 6 shows that total flavin in the liver of rats fed DAB is reduced as compared with the normal. The reduction of liver flavin contents is roughly paralleled with the depression of catalase.

Discussion

Hoshijima[1] has reported that the level of liver ferritin of mice decreases as the catalase activity of the same liver is reduced to below normal by toxohormone injections. In rats fed DAB, however, ferritin and hemosiderin in the liver increase as the catalase activity decreases below normal. Nevertheless, toxohormone and DAB had no direct effect on the crystalline catalase *in vitro*.

Liver ascorbic acid contents of rats fed DAB were also elevated above normal, while those of toxohormone treated mice were decreased.[16]

So the *in vivo* mechanism of action of toxohormone and DAB on the liver iron metabolism may be quite different from each other.

Reduction of flavin content in the liver of rats fed DAB agrees with the report of Suda.[17] But it is still uncertain how the flavin and metal ions might be separated from the metaloflavoprotein and removed from liver on this occasion.

Although the reason why the copper content in the liver is reduced in the precancerous state induced by DAB feeding is not explainable at present, these data together with those obtained by injections of toxohormone[1,16] will serve in the future to clarify what will happen at first in the liver iron metabolism due to DAB or toxohormone.

Summary

1. DAB had no effect on the crystalline beef liver catalase *in vitro*.

2. Hemosiderin, ferritin, and ascorbic acid contents in the liver of rats fed DAB for about 30 days were increased as compared with the normal, while catalase, copper, and riboflavin contents were decreased.

3. With the exception of ascorbic acid, the degree of changes of these components in the liver of rats fed DAB was in parallel with the depression of liver catalase.

The author wishes to thank Prof. S. Mizuhara and Mr. K. Okuda for their helpful suggestions. This investigation was aided in part by a research grant from the Ministry of Education.

References

1) Hoshijima, H., Gann, in press
2) Nakahara, W., and Fukuoka, F., Gann, **38**, 340 (1944)
3) Yanagizawa, I., Gann, **36**, 234 (1942)
4) Nakahara, W., and Fukuoka, F., Gann, **38**, 408 (1944)
5) Miyaji, T., Ozaki, K., and Nakazima, I., Igaku to Seibutugaku, **11**, 4 (1947)
6) Takeda, Y., and Hara, M., J. Biol. Chem., **214**, 657 (1955)
7) Mahler, H.R., Advances in Enzymol., **17**, 233 (1956)
8) Sarkar, N.K., and Sumner, J.B., Enzymologia, **14**, 280 (1951)
9) Chance, B., and Herbert, D., Biochem. J., **46**, 402 (1950)
10) Bonnichsen, R.K., Chance, B., and Theorell, H., Acta Chem. Scand., **1**, 685 (1947)
11) Yonekawa, M., and Konno, K., J. Biochem., **40**, 377 (1953)
12) Eden, A., and Green, H.H., Biochem. J., **34**, 1202 (1940)
13) Roe, J.H., and Oesterling, M.J., J. Biol. Chem., **152**, 511 (1944)
14) Yagi, K., Vitamins, **6**, 528 (1953)
15) Marston, H.R., Physiol. Rev., **32**, 66 (1952)
16) Hoshijima, H., unpublished data
17) Suda, M., Gann, **36**, 231 (1942)

要　旨

DAB 飼育白鼠の肝臓における変化について

第 1 報

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DAB で飼育した白鼠の肝臓における 1 カ月目の鉄代謝関連物質の変化をしらべたところ，hemosidein, ferritin, ascorbin 酸は正常白鼠に比し増加していたが，Catalase，銅及び総 riboflavin 量は減少していた。

Ascorbin 酸を除いては，上記成分の増減はすべて Catalase の減少と平行していた。

DAB は結晶 Catalase に対しては直接の抑制作用を示さなかった。

(文部省科学研究費による)

[GANN, 48, 249~253; October, 1957]

DEOXYALDOLASE ACTIVITY IN NEOPLASTIC TISSUES

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Concerning the origin of deoxyribose, Racker reported of deoxyaldolase which synthesizes deoxyribose-5-phosphate from acetaldehyde and 3-phosphoglyceraldehyde (1). He described the partial purification of this enzyme from *Escherichia coli* and also the distribution of this enzyme in mouse liver and thymus. McGeown and Malpress, too, reported of this enzyme in guinea pig liver (2). Another pathway was suggested by Lanning and Cohen as to the formation of deoxyribose in *Escherichia coli* infected with bacteriophage (3). Also, it was presumed that ribose moiety is transformed into deoxyribose moiety from Rose and Schweigert's experiment using labeled cytidine in mice (4). These pathways, however, have not been enzymatically defined. On the basis of the radioactivity distribution study among carbon atoms of deoxyribose moiety of DNA, Shreeve *et al.* recently stated that deoxyribose is formed via deoxyaldolase in regenerating liver of rats injected with glycine-1-C^{14} or glycine-2-C^{14} (5).

The origin of deoxyribose in neoplastic tissues is an interesting problem because of their rapid growth. We describe in this paper dexyaldolase activity in several neoplastic tissues, as we have not found any literature along this line except a quite recent paper by Shonk and Boxer (6), in which they determined this enzyme activity of liver and hepatoma.

EXPERIMENTAL

Liver of dba mouse, transplantable carcinoma of dba mouse (mammary tumor), NF-carcinoma (mammary carcinoma) of mouse, Yoshida ascites hepatoma of rat (Strain 130), and Rhodamine fibrosarcoma of rat were used. Tissues were dissected out and homogenized with 4 volumes of cold isotonic potassium chloride solution and centrifuged for ten minutes at 4,000 rpm in cold. Supernatant was used as enzyme extract.

Enzyme was assayed through the determination of deoxyribose with Dische's diphenylamine reagent after the incubation in the system as described by Racker. Tris (hydroxymethyl) aminomethane buffer was used instead of phosphate buffer, since Racker suggested that the former was better fitted than the latter for the

determination of crude enzyme extract. Crystalline aldolase was prepared from rabbit muscle and recrystallized by the method of Taylor *et al.* (7).

Results

Time relationship. The results of plotting the colorimetric readings against time in the experiments using enzyme extract of mouse liver are shown in Figure 1. It can been seen that the reaction velocity is almost constant for the first 45 minutes. On the basis of this finding, in all the following experiments for comparison of enzyme activity among various tissues, reactions were stopped after the incubation for 45 minutes.

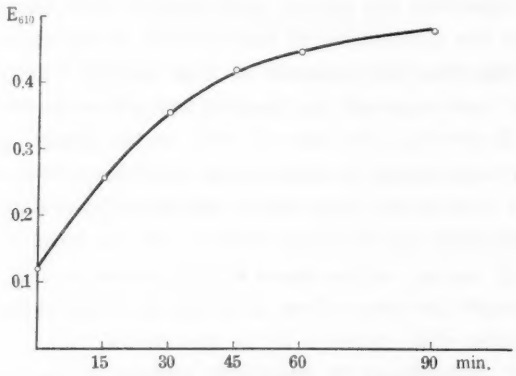

Fig. 1. Time Course of Deoxyribose Formation.

The system contained the following substances in a final volume of 2 ml: 60 μM of fructose-1, 6-diphosphate, 60 μM of acetaldehyde, about 500 μg of crystalline aldolase, 50 μM of trisaminomethane buffer, pH 7.2, and 0.6ml of enzyme extact.

2 Ml of 10% BCA was added and centrifuged. Deoxyribose content in 2 ml of supernatant was determined with 4 ml of Dische's reagent. Extinction of 0.100 corresponds to 0.153 mg of DNA.

Omission of substrates. The data of experiments, in which one substrate or component was omitted, are given in Table 1. In this experiment, the condition was almost the same as that of Figure 1. The ommission of aldolase had relatively little effect since crude tissue extract contained a considerable amount of aldolase.

Reaction Velocity versus Amount of Enzyme Extract. In Figure 2 are shown the results obtained when the colorimetric reading of the aliquot is plotted against the amount of enzyme extract from mouse liver and NF carcinoma tissues. The curves are linear for both extracts.

Table 1. Omission of substrate or component

	Reading
Complete system	0.460
Acetaldehyde omitted	0.030
HDP omitted	0.080
Aldolase omitted	0.350
Enzyme extract omitted	0.145

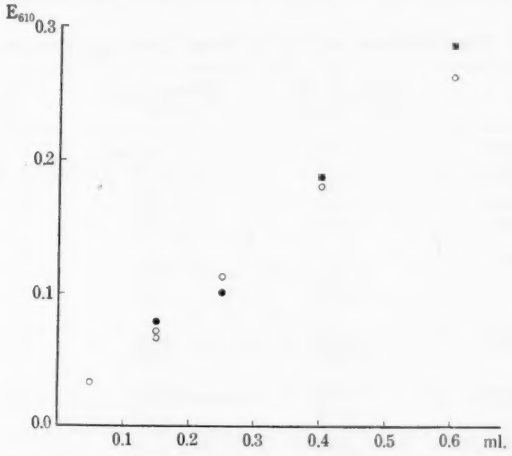

Fig. 2. Reaction Product and Enzyme Amount.
Experimental conditions were the same as described in the foot-note in Figure 1. 0.5Ml of enzyme extract of dba mouse liver and NF carcinoma contained 1.92 mg and 1.58 mg N respectively.

○ dba mouse liver
● NF carcinoma

Recovery of the Enzyme Activity in Extracts. In the case of mouse liver, the activity of enzyme extract showed 117 per cent of that of whole homogenate. This would indicate the presence of side reaction which promotes the degrading process of deoxyribose phosphate in whole homogenate of mouse liver. Hence, the comparison of enzyme activity among the various tissues was carried out using the enzyme extract.

Comparison of Enzyme Activity among Mouse Liver and Various Tumor Tissues. Since a high activity of mouse liver among mammalian tissues was noticed by Racker (1), we refered to the activity of mouse liver for comparison. Nitrogen was determined on the enzyme extract and the specific activity per uuit N was calculated. The data are given in Table 2. The deoxyaldolase in dba mouse mammary tumor is one-half of that of liver of normal dba mouse.

Hepatoma of rat, and Rhodamine sarcoma have three-fourths the activity of dba mouse liver and NF carcinoma of mouse has almost the same activity as that of dba mouse liver.

Deoxyaldolase Activity after the Whole Body Irradiation of Mice. In view of the fact that the synthesis of deoxyribose nucleic acid was inhibited by X-irradiation, we determined the deoxyaldoalse of liver of mice which received whole body Co^{60} gamma ray irradiation of 1,200 r. But as shown in Table 2, no decrease of deoxyaldolase activity was observed 3 hours after X-irradiation.

Table 2. Deoxyaldolase Activity of Mouse Liver and Various Tumors

Tissues	Reading of Colorimeter	Nitrogen in Extract	Specifc activity (arbitrary units)
dba Mouse liver	0.170 ϕ	1.41 mg	1.21
〃	0.178	1.41	1.26
〃	0.225	1.69	1.33
dba Mouse liver X irradiated	2.200	1.66	1.21
	0.190	1.60	1.19
dba Mammary tumor	0.076	1.21	0.63
	0.092	1.21	0.76
	0.040	1.16	0.35
Hepatoma (Rat)	0.128	1.16	1.10
	0.098	1.21	0.81
NF carcinoma	0.115	1.16	0.99
	0.141	1.16	1.22
Rhodamine sarcoma	0.081	1.11	0.73
	0.110	1.08	1.02

Experimental conditions were the same as those in foot-note in Figure 1.

Comments

From the above described results, it is clear that a considerable amount of deoxyaldolase is in various neoplastic tissues of mice and rats. Contrary to our expectation that neoplastic tissues would show very high activity of this enzyme because of the demand by deoxyribonucleic acid synthesis, various neoplastic tissues showed almost the same activity as or less activity than the normal mouse liver. Suffice it to record in this paper that a considerable amount of deoxyaldolase is contained in all the animal tumors examined and consequently probably in neoplastic tissues in general.

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References

1) Racker, E.: J. Biol. Chem., **196**, 347 (1952)
2) McGeown, M. G., and Malpress, F. H.: Nature, **170**, 575 (1952).
3) Lanning, M. C., and Cohen, C. C.: J. Biol. Chem., **216**, 413 (1955).
4) Rose, I. A., and Schweigert, B. S.: J. Biol. Chem., **202**, 635 (1955).
5) Shreeve, W., and Grossmann, A.: Fed. Proc., **16**, 248 (1957).
6) Shonk, C. E., and Boxer, G. E.: Fed. Proc., **16**, 247 (1957).
7) Taylor, J. F., Green, A. A., and Cori, G. T.: J. Biol. Chem., **173**, 591 (1948).

要　旨

癌組織のデオキシアルドラーゼ活性

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デオキシリボース生合成については Racker のデオキシアルドラーゼによって 3-phosphoglyceraldehyde とアセトアルデヒドから縮合する酵素が知られている。他のみちすぢも推測されているがまだ関与する酵素は確認されていない。

われわれは増殖の盛んな癌組織において，デオキシアルドラーゼ活性を測定することを試みた。数種のハツカネズミ，ダイコクネズミの移植性腫瘍は，Racker により哺乳動物の組織の中では活性が高いと報告されているハツカネズミの肝の約 2/3 の活性ないしほぼ等しい活性を示した。われわれは広く癌組織一般にこの酵素が存在するものと推定する。

(文部省科学研究費による)

[GANN, 48, 255~261 ; October, 1957]

SYNTHESIS OF DEOXYPENTOSE NUCLEIC ACID IN LIVER CELL OF RAT FOLLOWING PLASMOPHERESIS

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INTRODUCTION

There have been several investigations demonstrating that the high mitotic activity is frequently observed in the liver of animals bearing growths of proliferating tissues. In the tumor bearing animals, for example, the high mitotic activity and the remarkable incorporation of P^{32} and C^{14} precursors into deoxypentose nucleic acid (DNA) of liver cells have been reported[1,2,3,4]. Recently, the net synthesis of DNA (and multiplication) of liver cells in the tumor bearing animals has been observed[5]. Similar evidences have been found in the liver of pregnant animals[6,7,8,9]. It should be noticed here that the acceleration of DNA synthesis and of the mitotic activity may somewhat be specific in the liver cells.

It may be suggested that the synthesis of DNA and/or multiplication of liver cells is either 1) directly induced by the stimulating substances produced by the actively proliferating cells, such as the unfractionated factor reported by Malmgren[10], or 2) indirectly caused as results of the compensatory activity to make up for the deficiency of serum protein produced through the demand imposed by the proliferating tissue, the liver cell being the main, though not the only, locus of the synthesis of serum protein, specially albumin[11]. It has been recently found that the protein source for such proliferating cells may be the serum protein produced by the liver of the host animal[12]. Moreover, it is generally accepted that proteins of the non-proliferating as well as proliferating tissue cells are the metabolically active components in these cells. Therefore, supply of proteins from the serum is required not only by the proliferating cells but also by the non-proliferating cells, in order that the accumulation and renewal of the intracellular proteins be insured. If the consumption of the serum protein would greatly increase within the whole body, it should be expected that the activity of the protein synthesis in the liver is accelerated. In this case, whenever the necessary amount of the serum protein is beyond the limit of the ability of individual cells existing in a liver to produce it, the number

of liver cells might increase or the increase of almost all the cellular functions of the existing individual cells might occur in order to make adequate the protein synthesizing ability of the liver as a whole.

The purpose of the present investigation is to determine whether the multiplication of cells and/or DNA synthesis, which is necessary for cell division, in the liver of animals bearing actively poliferating tissues is owing to stimulating substances produced by the proliferating cells or to protein deficiency in the serum. As the first experiment of this investigation, the latter possibility was examined.

It was under such basic considerations that the following experiment was attempted.

Methods

Rats weighing 170–206 g were employed in the present investigation. Three ml of blood was removed from the mesenterial vein of the rat, anaethesized with ether gas, by the syringe which contained 0.04 mg heparine in 2 ml of the physiological saline. All of the erythrocytes in heparinized blood were washed twice with physiological saline in order to remove the serum protein. After removal of the serum protein, the washed erythrocytes were suspended in physiological saline and the final volume of the saline is adjusted to 3 ml. Then, these erythrocytes were slowly injected back into the mesenterial vein of the same rat. Thus the serum-protein deficient rats were obtained. One hour after the operation, 1 μc phosphate-P^{32} per g body weight was administrated by the subcutaneous injection. There was no evidence to show any remarkable degeneration of the washed and injected erythocytes. Twenty-four hours after P^{32} injection, rats were sarcrified, and the liver and kidney were immediately removed. These tissue samples of the experimental and control groups were then fractionated into DNA and PNA by a modification of the method of Amano *et al.*[13]

Diphenylamine reaction was employed for the estimation of DNA. The value of DNA-P was calculated on the basis of the phosphorus content of the standard DNA sample used in the calibration. Phosphorus content of PNA fraction was determined by Allen's method. Radioactivity of each fraction was measured with a Geiger-Müller tube (Scientific Research Co., Tokyo). P^{32} incorporation in respective compound was expressed in the relative specific activity (RSA) as given by the formula:

$$\text{RSA} = \frac{\text{cpm per mg DNA-P or PNA-P}}{\text{cpm of the total amount of injected } P^{32}.}$$

Results and Discussion

The results are summarized in Table 1. As is evident from the table, plasmopheresis results in high P^{32} incorporation in the liver, but not in the kidney, when P^{32} labeled phosphate was injected. P^{32} incorporation in the liver of the experimental animals was 5–10 times higher than that of the control animals and was no less than that of the regenerating livers (Exp. H 1, H 2 and H 3) 24 hours postoperatively, if the comparison was *roughly* attempted. But, the values of the specific activity of the experimental groups are approximately one-third of those of the regenerating livers in case of Exp. H 4, H 5 and H 6, which may be the highest values in the regenerating liver at 24 hours after P^{32} injection.

It should be remarked here that DNA is a metabolically inert component of the cell and the incorporation of P^{32} into DNA clearly indicates the net synthesis of DNA molecules, but not the intramolecular exchange of P^{32} and the turnover of DNA[14, 15, 16].

Table 1. P^{32} Incorporation into DNA-P and PNA-P of Liver and Kidney of Rats under Several Experimental Condtions.

Rat No.	Body weight (g)	Sex	Treatments	Injection of P^{32} (hours after treatment)	Hours after treatment	Relative Specific Activity ($\times 10^{-6}$)			
						DNA-P		PNA-P	
						Liver	Kidney	Liver	Kidney
E 1.	170	m	Ether anaethesia (for 45 min.), operation, and plasmopheresis	1	25	1839	—	2134	—
E 2.	202	m	〃	1	〃	1406	54	1400	799
E 3.	200	m	〃	1	〃	1366	81	1179	483
E 4.	195	m	〃	1	〃	1122	—	1157	—
C 1.	201	m	Mild anaethesia (for 0.5 -1 min.)	Immediately after	24	135	108	716	515
C 2.	192	m	〃	〃	〃	324	41	831	671
C 3.	189	f	〃	〃	〃	270	—	—	—
C 4.	198	m	〃	〃	〃	135	—	587	—
C 5.	183	f	〃	〃	〃	216	54	—	—
S 1.	202	m	Ether anaethesia (for 45 min.), and Sham-operation	〃	24	514	—	—	—
S 2.	188	m	〃	〃	〃	771	—	—	—
A 1.	189	f	Ether anaethesia (for 45 min.)	〃	24	581	—	—	—
H 1.	177	f	Partial hepatectomy	22.5	27	1041	—	—	—
H 2.	206	m	〃	23.5	〃	1122	—	1513	—
H 3.	173	f	〃	23.3	28.3	1595	—	—	—
H 4.	175	f	〃	24	48	6070	—	—	—
H 5.	189	f	〃	〃	〃	5138	—	—	—
H 6.	194	f	〃	〃	〃	3326	—	—	—

It has been widely accepted that P^{32} is incorporated rapidly into the precursors of DNA, with the maximal specific activity within 2 hours after injection, followed by a similarly rapid decrease[17]. Therefore, if the DNA synthesis in the liver tissues could show the maximal activity at the stage of 1~3 hours after plasmopheresis, the values of relative specific activity obtained under our experimental conditions would be the highest values. In the present investigation, however, the labeling of DNA at various times postoperatively was not carried out. Under such circumstances, an accurate comparison of such data with the incorporation of P^{32} into DNA of the regenerating liver following partial hepatectomy may not be possible.

Although anaethesia results in a slight incorporation of P^{32} into DNA, it is certain that the half or more of the individual value of RSA obtained in the experimental groups is to be attributed to the plasmopheresis.

Table 2. P^{32} Incorporation into DNA-P of Liver and Tumor of Rats bearing Rhodamine Sarcoma.

Rat No.	Body weight including tumor (g)	Sex	Tumor weight (g)	Injection of P^{32} (days after tumor transplantation)	Hours after injection of P^{32}	Relative Specific Activity ($X10^{-6}$)	
						Liver	Tumor
T 1.	210	m	44.2	23	24	730	1325
T 2.	201	m	46.3	23	24	379	1879
T 3.	167	f	7.8	18	24	419	—

It should be noted here that P^{32} incorporation into DNA of the liver following plasmopheresis was much higher than that of the livers of the animals bearing transplantable rhodamine sarcoma, as seen in Table 2. The question arises why a higher incorporation in the tumor bearing animals was not observed in the present investigation. There are two possibilities, of which the first may be of importance. That is, protein deficiency in the tumor bearing animals may be mild and continuous, but not drastic and temporary as in the case of experimental plasmopheresis. If so, the *rate* of increase of DNA synthetic activity would not be expected to be remarkably high. Theoretically the low incorporation may also be attributable to the marked deterioration of the host animal towards the last stage of the tumor growth but the tumor bearing animals we used were not in such a stage.

Mortreuil *et al.*[2] reported recently that the the liver of the animal, from which a large volume of blood was removed, could incorporate a somewhat large amount of P^{32} into their DNA and concluded that the high rate of incorporation of P^{32} into DNA in the liver of the tumor bearing animal is not owing to some substances produced by the tumor cells as expected from the findings by Malmgren[10]. However, their experiment still leaves a doubt as

to whether the deficiency of the serum protein did cause the induction of DNA synthesis in liver cells or not. The removal of a large amount of blood, including erythrocytes, inevitably induces very active regenerative proliferation of bone marrow, and thus introduces the possibility of certain substances produced by the actively proliferating cell (bone marrow cells) directly causing the increased DNA synthesis in the liver cell. The result obtained by us in the present investigation adequately rules out that possibility, and shows that the fact observed by Mortreuil *et al.* can fairly be explained on the ground of serum protein deficiency.

In conclusion, the results obtained here strongly support the second possibility which is mentioned in the introduction of this report. It does not mean, however, that the first possibility, though remote, is perfectly excluded.

In this connection, it is worth referring to the experiments by Campbell *et al.*[6,18] They found that there is a rise of DNA and RNA contents of the liver in pregnant mice and rats and considered that the excess RNA may be due to a factor or factors produced by placenta, particularly because the rise of DNA content corresponds to the increase in maternal weight plus the uterine contents. Such evidences are of just the same nature as those of tumor bearing animals. Our hypothesis clearly accounts for the observed increases of nucleic acids contents of livers in pregnant animals and it renders unnecessary to consider the possible action of the placental factor or factors on the maternal liver.

From a broader point of view the hypothesis proposed in this report may be concerned with the control of the cell population in the liver. The synthesis of albumin must be considered a characteristic function of the liver. Thus, generally speaking, the liver cell population might be partially controlled by the dynamic interrelation between the consumption and the production of some kind of serum protein, probably albumin.

Finally, we would like to add that, as seen in Table 1, an increased incorporation of P^{32} into PNA of the liver in the experimental group may be of paticular importance in relation to the production of serum proteins, since the close relation between PNA and the protein synthesis is now widely accepted.[19,20,21]

Summary

Plasmopheresis accelerated the synthetic activity of DNA in liver cells of the rat, but not in kidney cells. The hypothesis that the serum protein deficiency causes the induction of DNA synthesis in the liver, was supported by such a result. The possible control of liver cell population was also discussed from this point of view.

Acknowledgements

It is pleasure to acknowledge our indebtedness to Dr. W. Nakahara, Director of Cancer Institute, for his encouragement and valuable advice throughout this investigation. Thanks are further due to Prof. A. Sibatani, Department of Cytochemistry, Yamaguti Medical School, for his valuable advice and suggestion. This work was aided in part by the Scientific Research Fund of the Ministry of Education given to the Research Group on "Biochemistry of Cancer."

References

1) Annau; E., Manginelli, A., and Roth, A., Cancer Research, **11,** 304 (1951).
2) Mortreuil, M., and Khouvine, Y., Bull. Soc. Chim. Biol., **38,** 951 (1956).
3) Payne, A. H., Kelly, L. S., Beach, G., and Jones, H. B., Cancer Research, **12,** 426 (1952).
4) Payne, A. H., Kelly, L. S., and White, M. R., Cancer Research, **12,** 65 (1952).
5) Naora, H., and Naora, H., to be published.
6) Campbell, R. M., and Kosterlitz, H. W., J. Endocrinol., **9,** 45 (1953).
7) Kosterlitz, H. W., and Campbell, R. M., Nature, **160,** 675 (1949).
8) Kelly, L. S., Payne, A. H., White, M. R., and Jones, H. B., Cancer Research, **11,** 694 (1951).
9) Smellie, R. M. S., McIndoe, W. M., Lagan, R., Davidson, J. N., and Dawson, I. M., Biochem. J., **54,** 280 (1953).
10) Malmgren, R. A., Cancer Research, **16,** 233 (1956).
11) Miller, L. C., Bly, C. G., Watson, M. L., and Bale, W. F., J. Exp. Med., **54,** 431 (1951).
12) Babson, A. L., Biochim. Biophys. Acta, **20,** 418 (1956).
13) Amano, M., Kihara, H. K., and Sibatani, A., Biochim. Biophys. Acta, in press.
14) Kihara, H K., Amano, M., and Sibatani, A., Biochim. Biophys. Acta, **21,** 489 (1956).
15) Siminovitch, L., and Graham, A. F., J. Histochem. Cytochem., **4,** 508 (1956).
16) Daoust, R., Leblond, C. P., Nadler, N. J., and Enesco, M., J. Biol. Chem., **221,** 727 (1956).
17) Barnum, C. P., and Huseby, R. A., Arch. Biochem., **29,** 7 (1950).
18) Campbell, R. M., and Kosterlitz, H. W., J. Endocrinol., **6,** 171 (1949).
19) Brachet, J., The Nucleic Acids, **2,** 475 (1955).
20) Gale, E. F., In Enzymes: Units of Biological Structure and Function, ed. by Gaebler, O. H., 49 (1956).
21) Moyson, F., and Steens-Lievens, A., Biochim. Biophys. Acta, **21,** 500 (1956).

要　旨

Plasmopheresis による肝細胞 DNA 合成機能の誘発について

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動物体内の一部に非常に増殖しつつある組織をもっている動物の肝では，DNA の合成が盛んに行われ，有糸分裂像がしばしば見出されるということが一般にみとめられている。これには，①. 増殖性細胞 (例えば癌細胞) により産生される Stimulating substances による場合及び，②. 増殖性細胞の成長増殖のために動物体内で血清蛋白の欠乏が生ずる，それの補償的な意味で，血清蛋白の製造所である肝細胞の増殖 (あるいはそれに匹敵する現象) が起るという2個の可能性がある。ここでは後者の可能性にたって，人工的に生ぜしめた血清蛋白欠乏ネズミに P^{32} を皮下注射して，肝及び腎の DNA-P 及び PNA-P へのとりこみをみた。

Plasmopheresis によって肝の DNA 合成能は非常に高められ，その合成能は，再生肝にみられる合成能に比較できる程度のものである。しかしながら，この場合，腎の DNA 合成能は誘発されない。

このような事実は，さきにのべた ② の可能性を支持するものである。

なお，肝における細胞数の調節機構についても，このような見地に立って，若干，論議した。

(文部省科学研究費による)

[GANN, 48, 263~270; October, 1957]

ANTI-CANCER ACTION OF 6-CARBOXY-4-NITROQUINOLINE 1-OXIDE

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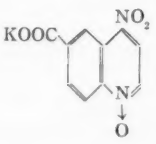
and SACHIKO SUZUKI
(Pharmaceutical Institute, Medical Faculty, University of Tokyo)

In a previous paper we reported the results of screening a large series of quinoline derivatives for their anti-cancer actions, based on *in vitro* as well as *in vivo* tests (1), and showed that 4-nitroquinoline 1-oxide and certain of its derivatives are markedly active. These substances not only killed cancer cells *in vitro* at a very low concentration but also produced a notable prolongation of the survival period of the mice inoculated with Ehrlich ascites carcinoma.

From a practical point of view one drawback to these quinoline derivatives was their insolubility in water, which forced us to use suspensions of the substances, with disadvantages inherent in such practice. In order to overcome this difficulty we have attempted to produce a water-soluble derivative of 4-nitroquinoline 1-oxide, and this objective has been attained by the successful synthesis of 6-carboxy-4-nitroquinoline 1-oxide alkaline salts of which are soluble in water. In the present paper we report our experimental data on toxicity, cancericidal action *in vitro*, effect on Ehrlich ascites carcinoma *in vivo*, and inhibiting action on glycolysis of tumor cells and on crystalline aldolase, of this newly synthesized water-soluble derivative.

Material



Potassium salt of 6-carboxy-4-nitroquinoline 1-oxide used in this series of experiments was prepared by one of us (Suzuki) at the Pharmaceutical Institute, University of Tokyo, under the guidance of Professor Eiji Ochiai. It was needle crystals of very light weight, lemon yellow in color, decomosing at 280~290°C. The details of the method for the synthesis of this substance will be published later elsewhere.

Toxicity

Compared to the related but water-insoluble derivatives, the salt of 6-carboxy-4-nitroquino-line 1-oxide was found to be very much less toxic to mice. The results

Table 1. Toxicity test expressed in the ratio of number of death over total number of mice

Time after injection	Doses: mg per kg body weight (Injected intraperitoneally)			
	300	500	600	700
24 hrs.		2/4	1/4	4/4
48 hrs.			3/4	
3 days			4/4	
4 days		2/4		
5 days	0/4	4/4		

of toxicity test, as shown in Table 1, indicated that LD_{50} for this substance may be estimated to be about 550mg per kg ofb ody wight.

It may be recalled that LD_{50} for 4-nitroquinoline 1-oxise, as tested previously, was 70 mg, 65 mg, 50 mg, 45 mg, 40 mg, and 30 mg at 24 hrs., 48, hrs., 3 days, 4 days and 5 days, respectively, varying no doubt due to the relatively slow absorption. Approximately, therefore, the toxicity of the 6-carboxy derivative may be said to be 1/10 of that of 4-nitroquinoline 1-oxide.

Cancericidal Action In Vitro

The simple and practical technique used for the preliminary screening in previous studies was followed. Ten day old well growing subcutaneous grafts of mouse sarcoma 180 were used. Approximately the same amount of the freshly excised tumor tissue was cut up into fragments of about 1 mm diameter, was distributed as evenly as possible into four Petri dishes containing 5cc of 0.05%, 0.005%, 0.0005% and 0.0% (blank control) of 6-carboxy-4-nitroquinoline 1-oxide potassium salt in physiological salt solution and well mixed. The 0.05% solution was pH 5. The four Petri dishes were allowed to stand at the temperature of 4—6°C for 24 hours, after which the tumor tissue fragments were implanted by means of trocar into the subcutaneous tissue at four different sites of one mouse, using four mice in a test. The cancericidal action of the substance at three different dilutions was thus determined by the outcome of the implantation. The results were as follows (Table 2):

Table 2. Effect of potassium salt of 6-carboxy-4-nitroquinoline 1-oxide on the survival *in vitro* of sarcoma 180

	At the concentration of			
	0.05%	0.005%	0.0005%	0%
No. tumor / No. implantations	0/4	4/4	4/4	4/4

According to our previous experiments 4-nitroquinoline 1-oxide completely robbed the viability of tumor tissue at the concentration of 0.01%. At 0.005% 4 tumors were produced out of 6 implantations. The cancericidal action *in vitro* of the 6-carboxy derivative may be estimated to fall somehwere between 1/5 to 1/10 of that of 4-nitroquinoline 1-oxide.

Effect In Vivo on Ehrlich Ascites Carcinoma

Ascites fluid of Ehrlich mouse carcinoma was freshly aspirated one week after the inoculation, and was injected in 0.05cc doses intraperitoneally into five groups of normal mice, all males, each of about the same body weight. The tumor cell content of the ascites fluid was estimated to be 1,000,000 to 1,500,000 per cc.

Treatment with intraperitoneal injections of potassium salt of 6-carboxy-4-nitroquinoline 1-oxide was started 24 hours after the ascites injection, and was repeated daily for 7 consecutive days. The substance was administered dissolved in physiological salt solution at three different dosage levels, namely: 100 mg per kg, 125 mg per kg, and 140 mg per kg. per injection. For comparison, and also as a

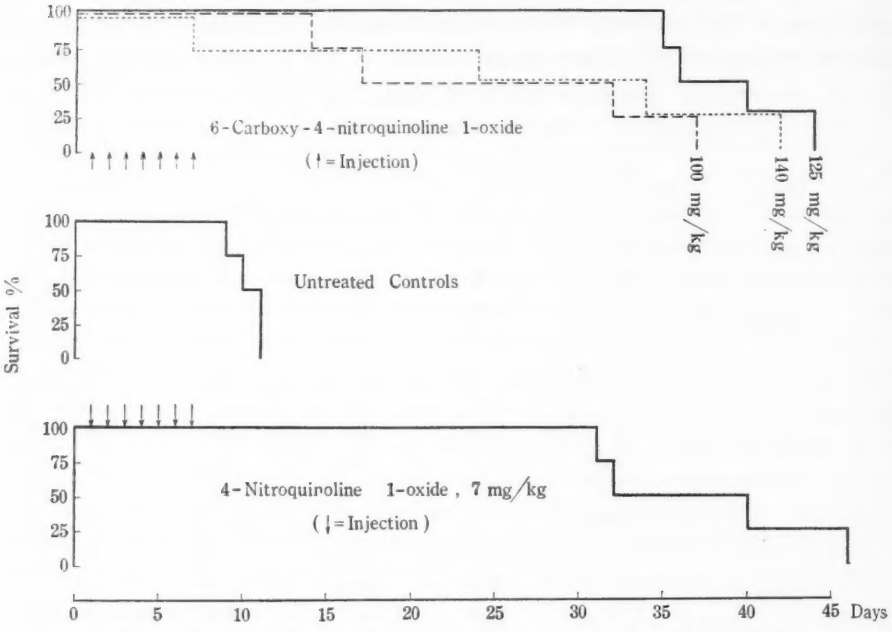
Table 3. Effect of 6-carboxy-4-nitroquinoline 1-oxide on Ehrlich ascites carcinoma *In Vivo*

Doses	Mouse No.	Body weight, g			Survivial Days
		Initial	After 1 week	Difference	
100 mg per kg	1	16.3	15.5	−0.3	37
	2	17.5	14.0	−3.5	14
	3	18.0	16.0	−2.0	32
	4	19.5	15.0	−4.5	17
	(Average)			(−2.7)	(25)
125 mg per kg	1	16.5	17.5	+1.0	40
	2	17.5	16.5	−1.0	36
	3	18.5	19.5	+1.0	44
	4	18.0	17.5	−0.5	35
	(Average)			(+0.1)	(38.7)
140 mg per kg	1	16.9	16.0	−0.9	42
	2	17.0	15.2	−1.8	34
	3	19.0	18.7	−0.3	24
	4	19.3	15.0	−4.3	7
	(Average)			(−1.8)	(26.7)
0 mg	1	16.5	18.0	+1.5	11
	2	17.3	19.0	+1.7	11
	3	18.7	18.0	−0.7	10
	4	18.0	20.0	+2.0	9
	(Average)			(+1.1)	(10.2)
4-Nitroquinoline 1-oxide. 7 mg per kg	1	16.4	17.5	+1.1	32
	2	17.5	17.0	−0.5	46
	3	19.5	14.5	−5.0	31
	4	17.9	17.5	−0.4	40
	(Average)				(37.2)

sort of positive control, one group was treated under the identical conditions with the optimum dose (7 mg per kg) of 4-nitroquinoline 1-oxide.

In judging the effect of the treatment, weekly body weight changes, state of abdominal distention, as well as microscopical findings on ascites were taken into consideration, but the number of days of survival was used as by far the most important criterion. The results of the experiment are shown in Table 3, and illustrated in Chart 1.

Chart 1. Effect of 6-carboxy-4-nitroquinoline 1-oxide on the survival of mice inoculated with Ehrlich ascites carcinoma



From the above results the optimum dose of the 6-carboxy compound may be said to be about 125 mg per kg per injection, which is about 20 times that of 4-nitroquinoline 1-oxide. It is of interest to note that, compared to the latter, the former is about 1/10 as toxic as well as cancericidal *in vitro*.

When the treatment with optimum doses was started 24 hours after the tumor inoculation there seems to result a complete cure of ascites tumors as far as can be judged from the lack of abdominal distention and from microscopical findings on the peritoneal fluid. Unfortunately, however, the repeated intraperitoneal injections in mice bearing ascites carcinoma inadvertently give rise to subcutaneous tumors, and it is from such secondarily produced tumors that the treated mice eventually die. Evidently, 6-carboxy-4-nitroquinoline 1-oxide treatment as

carried out in the above experiments, is inadequate to suppress the production and growth of secondary subcutaneous tumors. Whether established solid tumors can be markedly influenced by more intensive treatment remains to be determined by further experiments.

Inhibition of Tumor Cell Glycolysis

In order to obtain some insight into the biochemical mechanism of the anti-cancer action of quinoline derivatives we previously investigated the effect of these substances on the glycolytic activity of Ehrlich carcinoma cells, and found a very satisfactory parallelism to exist between the anti-cancer and glycolysis-inhibiting actions (2). The inhibition of glycolysis may be of great significance in the mode of action of any cancericidal substance, since the energy yielded from glycolysis is now known to be utilized for many important synthetic processes in the metabolism of cancer cells.

We examined the ability of 6-carboxy-4-nitroquinoline 1-oxide to inhibit the glycolytic activity of Ehrlich ascites cells after the manner of our previuos experiments. The ascitic fluid, taken out one week after the inoculation, was freed of contaminating red cells and tumor cells were re-suspended at a suitable concentration in Krebs-Ringer bicarbonate buffer solution. Warburg vessels were set up, each containing glucose in the final concentration of 200mg/dl, $1.0\text{-}1.6\times10^{7}$ tumor cells, and potassium salt of 6-carboxy-4-nitroquinoline 1-oxide in the same buffer solution to make various final concentrations, the total amount of the mixture being 3 ml. A mixture of 95% nitrogen and 5% carbon dioxide was used for gas phase.

The results obtained showed that potassium salt of 6-carboxy-4-nitroquinoline 1-oxide produced about 50% inhibition at the concentration of 1×10^{-2} M, in contrast to ca. 60% inhibition brought about by 4-nitroquinoline 1-oxide at 4×10^{-4} M. This difference in the effective concentration between the two substances is well correlated to the difference in their toxicity and anti-cancer activity, both *in vitro* and *in vivo*.

Inhibition of Aldolase Activity

Since aldolase, which splits hexose into two trioses, is an important part of the chain of glycolytic process, we next tested the inhibiting action on the activity of this enzyme not only of 6-carboxy-4-nitriquinoline 1-oxide, but also, for comparison, of 4-nitroquinoline 1-oxide, which is strongly cancericidal, and of quinoline 1-oxide, which is practically inert.

The sample of aldolase used was a crystalline preparation isolated from rabbit skeletal muscle, according to the method of Taylor *et al.* (3), twice recrystallized

from $(NH_4)_2SO_4$. The enzyme activity was measured following the method of Dounce *et al.* (4), using disodium fructose diphosphate as substate and determining the triose phosphate formed. The triose phosphate was converted by means of hot concentrated sulphuric acid to acetaldehyde, which was then measured by color produced with p-hydroxydiphenyl.

The results showed that potassium salt of 6-carboxy-4-nitroquinoline 1-oxide produced about 60% inhibition, 4-nitroquinoline 1-oxide about 50% inhibition, and quinoline 1-oxide less than 20% inhibition, all at the same final concentration of 10^{-3} M.

The inhibiting actions on aldolase activity of the three derivatives, as seen from these results, are surprisingly little-different, which is in marked contrast to the great differences in their other actions. The lack of any striking difference here is especially notable when considered in relation to the difference in the glycolytic inhibition. It is possible that these quinoline derivatives attack a part other than aldolase of the chain of glycolytic reaction. However, experimental conditions, using crytstalline enzyme on one hand and living tumor cells on the other, are so different that no hasty conclusion can be drawn. We simply record here our findings for what they are worth, reserving for future more detailed investigations into the exact mechanism of the glycolytic inhibition by these quinoline derivatives.

Comments

Experimental data reported in this paper show that the water soluble alkali salt of 6-carboxy derivative of 4-nitroquinoline 1-oxide is cancericidal ***in vitro*** at a much higher concentration than the water insoluble derivatives. This reduced activity, however, is compensated for by a corresponding decrease in the toxicity, and in appropriately larger doses it is possible to obtain a comparable effect on Ehrlich ascites carcinoma ***in vivo***.

One important point which should be mentioned here is the recently discovered fact that 4-nitroquinoline 1-oxide, and probably other water-insoluble derivatives also, are highly carcinogenic when applied on the skin of mice (5). These substances thus form outstanding examples of the postulate of Haddow that chemicals which cure cancer may also cause it and ***vice versa***. Whether the water-soluble alkali salt of 6-carboxy-4-nitroquinoline 1-oxide is also carcinogenic or not remains yet to be determined, but in view of the almost universal insolubility in water of really powerful carcinogens so far discovered, it may not be unreasonable to expect that the 6-carboxy derivative may be only weakly carcinogenic, if at all.

Summary

Potassium salt of 6-carboxy-4-nitroquinoline 1-oxide was tested as to its toxicity, cancericidal action *in vitro*, and effect *in vivo* on Ehrlich ascites carcinoma. Compared to the water-insoluble derivatives previously studied, this water-soluble 6-carboxy compound was found to be about 1/10 as toxic and as effective in cancericidal action *in vitro*. In *in vivo* tests, the substance in appropriate doses completely destroyed Ehrlich ascites carcinoma when the treatment was started 24 hours after the tumor inoculation. The optimum doses were found to be about twenty times that of 4-nitroquinoline 1-oxide.

It was also found that 6-carboxy-4-nitroquinoline 1-oxide inhibits the glycolytic action of Ehrlich carcinoma cells *in vitro* at about twenty times the concentration which is necessary for 4-nitroquinoline 1-oxide. However, no corresponding difference was recognized between the two substances as to their power of inhibiting the activity of crystalline aldolase.

The authors wish to express their cordial appreciation to Professor Eiji Ochiai, Pharmaceutical Institute, University of Tokyo, and to Doctor Waro Nakahara, Director of Cancer Institute, for their guidance and support.

References

1) Sakai, S., Minoda, K., Saito, G., Akagi, S., Ueno, A., and Fukuoka, F.: Gann, 46 : 605 (1955).
2) Fukuoka, F., Sugimura, T., and Sakai, S.: Gann, 48 : 65 (1957).
3) Taylor, J. F., Green, A. A., and Cori, G. T.: Jour. Biol. Chem., 173 : 591 (1948).
4) Dounce, A. L., and Beyer, G. T.: Jour. Biol. Chem., 173 : 159 (1948)
5) Nakahara, W., Fukuoka, F., and Sugimura, T.: Gann, 48 : 129 (1957)

要　旨

6-Carboxy-4-Nitroquinoline 1-Oxide の抗癌性について

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鈴木幸子 (東京大学　医学部　薬学科)

われわれは多数のキノリン誘導体の制癌作用についての実験を行い，これら誘導体中 4-nitroquinoline 1-oxide，4-nitro-2-alkylquinoline 1-oxide，6-bromo-4-nitroquinoline 1-oxide，等，4位にニトロ基を有する quinoline 1-oxide 型の物が最も強い制癌作用を有することを報告している。しかるにこれらはいずれも水に難溶であったので，6 位にカルボキシル基を入れ (すなわち 6-carboxy-4-nitroquinoline 1-oxide)，これを K 塩とすることにより水溶性とした，今回は主としてこの物質の制癌性について，もとの 4-nitroquinoline-1-oxide その他と毒性，制癌性及びそれらの作用機作について比較実験した結果を報告した。

LD_{50} は前者 70mg/kg に比し 650/kg で毒性は約 1/10 近く，in vitro の殺癌効果は 1/10 でほぼ同じであり，延命効果は 125mg/kg で前者の 7/kg の場合に匹敵した。次に解糖に対する態度は，前者 4×10^{-4} M で 60% 阻害を示し，本物質は 1×10^{-2} M で約 50% 阻害を示す．したがって濃度比は in vitro 及び in vivo の場合のそれに等しいといえよう。

さらに今回は，作用機作の一連の実験の一つとして，アルドラーゼ阻害について，これら二物質以外全く制癌性のない quinoline 1-oxide をも含めて，比較したところ，10^{-3} M で nitroquinoline 1-oxide では 50%，6-carboxyquinoline-uoxide では 60% 阻害を示し，全く抗癌性のない quinoline 1-oxide ではわずか 20% の阻害であった。

(文部省科学研究費による)

[GANN, 48, 271~276; October, 1957]

INHIBITION OF P^{32} INCORPORATION IN VITRO INTO NUCLEIC ACIDS OF EHRLICH CARCINOMA CELLS BY QUINOLINE DERIVATIVES

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Our interest in the anti-cancer action of certain quinoline derivatives[1] led us to undertake some investigations concerning the biochemical mechanisms involved in their actions. In view of the crucial importance of glycolysis as the energy-yielding process for cancer cells we previously tested the effect of quinoline derivatives on the glycolytic activity of Ehrlich ascites carcinoma cells, and found a very satisfactory parallelism existing between the anti-cancer and glycolysis-inhibiting actions.[2] As a second step along this line of study, we have now taken up the effect on nucleic acid synthesis, since the inhibition of nucleic acid (especially DNA) synthesis is reported to be an important mode of action of certain types of anti-cancer agents[3,4,5,6,7,8]

In the present paper is reported that part of our experiments which deals with the effect of selected quinoline derivatives on the incorporation of P^{32} into nucleic acids of cancer cells *in vitro*.

Meterial and Methods

Ehrlich ascites carcinoma cells were used throughout the experiments. The tumor cells were obtained from mice 5 days after intraperitoneal transplantation, and the pooled cells were washed twice with Krebs-Ringer bicarbonate solution by centrifugation in order to remove contaminating blood cell. The cells, approximataly 3×10^9 in number, were suspended in 20 ml of Krebs-Ringer bicarbonate solution (pH 7.3), with the addition of 5 μc/ml of radioactive orthophospate.

After adding suitable amounts of quinoline derivatives to be tested, with adequate controls, the mixtures were incubated at 37°C for 1 hour under the aerobic conditions (O_2 : CO_2=95 : 5).

After the incubation period the tumor cells were recovered by centrifugation and DNA and RNA fractions were separated by the procedure proposed by Amano, Kihara and Sibatani[9], a modifications of methods of Schmidt, Thannhauser and others, which may be described in detail as follows:

The cells recovered by centrifugation were treated with 30 ml of 10% trichloroacetic acid and the precipitate, after being washed with 10% trichloroacetic acid,

was evenly suspended in 4 ml of distilled water, and then precipitated by adding 30 ml of ethanol. The precipitate was washed with 20~30 ml of ethanol saturated with sodium acetate and was suspended in ethanol saturated with sodium acetate. After standing overnight, the precipitate was washed with ethanol-chloroform (3 : 1), with ethanol-ether (3 : 1), and then with acetone, and dried.

The dried precipitate was heated at 100°C for 30 minutes in the mixture of 5ml of veronal buffered saline (pH 7.4) and 1 ml of 5% dodecyl sulphate, and, after adding 0.3g of sodium chloride per 5ml to make 1M, it was again heated at 100°C for 30 minutes.

Supernatant was now recovered. Precipitate was washed again with 2ml of 1M solution of sodium chloride, and the supernatant was combined with the first supernatant.

To the combined supernatant was added 2 ml of chloroform, well shaken, and then centrifuged at 3000 rpm for 20~30 minutes. The upper layer was carefully removed, and to it was added 1 volume of acetone and 2 volumes of ethanol. When precipitate was well formed it was separated by centrifugation, dissolved in 3 ml of distilled water and reprecipitated by adding 7ml of ethanol. Precipitate was again dissolved in 4 ml of distilled water and 1 ml of 1 N hydrochloric acid.

The precipitate formed was washed with 0.2 N hydrochloric acid, and again with ethanol, and then dried. The dried precipitate was warmed in 2ml of 0.1N sodidm hydroxide solution at 80°C for 10 minutes, and then ice-cooled. To the mixture was now added 0.5 ml of 1 N hydrochloric acid and then 0.3 ml of 50% trichloroacetic acid, and centrifuged.

Supernatant is PNA fraction (shaken with chlorofrom).

Precipitate was heated at 80°C for 20 minutes in 2 ml of 0.1 N sodium hydroxide solution, and then 0.5 ml of 1 N hydrochloric acid added to produce precipitate at 0~3°C. Precipitate was then heated at 90°C for 15 minutes in 2.5 ml of 10% trichloroacetic acid. Supernatant thus obtained is DNA fraction (shaken with chlorofrom).

Specific activity of the purified nucleic acids were expressed as counts per minute per mg of DNA-P or PNA-P.

Effect of 4-Nitroquinolin 1-Oxide and 6-Carboxy-4-Nitroquinoline 1-Oxide in Optimal Anti-Cancer Doses

In the first experiment was investigated the effect on P^{32} incorporation into nucleic acids of Ehrlich carcinoma cells of 4-nitroquinoline 1-oxide and 6-carboxy-4-nitroquinoline 1-oxide potassium salt. The latter is a recently synthesized water soluble derivative which is much less toxic and correspondingly less active against cancer than the former[10]. These substances were used in amounts

corresponding to the optimal anti-cancer dose of each. The insolubility of 4-nitroquinoline 1-oxide necessitated the introduction of propylene glycol as solvent.

A set of four reaction mixtures was set up, each containing tumor cells and P^{32}-labelled orthophosphate in Krebs-Ringer bicarbonate solution as already described. One was reserved as blank control without further addition; to the second was added 0.4 ml of propylene glycol; to the third 1.4mg of 4-nitroquinoline 1-oxide dissolved in 0.4 ml of propylene glycol; and to the fourth 25 mg of 6-carboxy-4-nitroquinoline 1-oxide potassium salt.

After incubation at 37°C for 1 hour, nucleic acid fractions were isolated and their specific activity determined in the manner as described above.

The following results were obtained (Table 1):

Tabl 1. Effect of 4-nitroquinoline 1-oxide and 6-carboxy-4-nitroquinoline 1-oxide

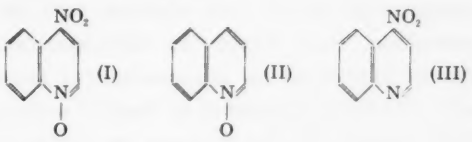
	Specific Activity		Relative Incorporation			
	DNA-P	PNA-P	DNA-P		PNA-P	
Blank control	707	9908	100	—	100	—
Propylene glycol control	580	6402	82	100	65	100
4-Nitroquinoline 1-oxide and propylene glycol	72	1226	10	12	12	19
6-Carboxy-4-nitroquinoline 1-oxide	87	2058	12	—	21	—

A marked inhibition of P^{32} incorporation into both DNA-P and PNA-P by the two quinoline derivatives is clearly demonstrated. It is of special interest that the two derivatives in amounts corresponding to their optimal anti-cancer doses, produced nearly the same degree of inhibition.

Effect of Inactive Derivatives as Compared to That of 4-Nitroquinoline 1-oxide

In the second experiment an attempt was made to compare the effect of actively anti-cancerous and inactive quinoline derivatives on P^{32} incorporation into nucleic acids. 4-Nitroquinoline 1-oxide (I) was used as an example of active substance, and as representatives of inactive derivatives quinoline 1-oxide (II) and 4-nitroquinoline (III) were selected. (See our previous papers (1, 2).

These substances were added to the reaction mixture on the same molar basis, namely, 1.4 mg/20 ml for (I), 1.3 mg/20 ml for (II) and (III). They were dissolved in 0.4 ml of propylene glycol. All other experimental conditions were



identical with those of the preceding experiment.

The results obtained are shown in Table 2.

Table 2. Effect of 4-nitroquinoline 1-oxide, quinoline 1-oxide, and 4-nitroquinoline

	Specific Activity		Relative Incorporation	
	DNA-P	PNA-P	DNA-P	PNA-P
Control (with propylene glycol)	15107	116053	100	100
4-Nitroquinoline 1-oxide	295	1458	2	1
Quinoline 1-oxide	14231	111114	94	96
4-Nitroquinoline	322	1214	2	1

The above data confirmed the marked inhibition of P^{32} incorporation by 4-nitroquinoline 1-oxide, but somewhat to our surprise demonstrated that two inactive derivatives strikingly differ in their inhibiting effect on the incorporation: 4-nitroquinoline showing the inhibition of identical degree with 4-nitroquinoline 1-oxide, and qunoline 1-oxide inhibiting practically not at all.

It would seem that the inhibition of P^{32} incorporation into nucleic acids by these quinoline derivatives depends upon the presence of nitro radical at the position 4, and is not directly related to their anti-cancer activities.

The marked difference in the specific activities in controls between this and the preceding experiments is probably explainable on the basis of possiblly different physiological states of the tumor cells used. Tumor cells used in the first experiment showed exceptionally low mitotic rate. This, however, does not interfere in any way with the interpretation of the results.

Discussion

The general morphological changes in the Ehrlich carcinoma ascites during the course of the treatment with actively anti-cancerous quinoline derivatives have been known to us for some time. These changes consisted of a marked diminution in the number of tumor cells, accompanied with the appearance of numerous leucocytes, in contrast to the steady increase of tumor cells, almost without leucocytic reaction, in untreated cases. However, such obvious signs of cell degeneration as nuclear fragmentation, pyknosis, etc., have not been conspicuous. It has also been clear that the effect of quinolines does not involve any readily recognizable specific cell changes such as chromosomal aberrations, arrested metaphase, etc. From the microspectrophotometric examination of Feulgen stanied specimens an impression was gained that a high percent of octoploid cells (or Class III proposed by Swift[11]) appear, and also cells with abnormally low DNA content, but our data are yet inadequate to base definite conclusions upon.

Effect on the synthesis of nucleic acids may be by far the more important

problem in elucidating the mechanism of anti-cancer action of quinolines. However, at the present stage of our investigation it is premature to draw any sweeping conclusion either as to the effect of quinoline derivatives on the nucleic acid synthesis or regarding the role of the inhibition of nucleic acid synthesis in the biochemical mechanisim of the anti-cancer action of these substances.

Our data concerning the inhibition of P^{32} incorporation into nucleic acids of Ehrlich carcinoma cells *in vitro* are presented here merely for what they are worth, pending much further studies which are needed to clarify the entire problem.

Summary

Effect on the *in vitro* incorporation of P^{32} into nucleic acids of Ehrlich carcinoma cells was tested with two actively anti-cancerous and two inactive quinoline derivatives.

It was found that 4-nitroquinoline 1-oxide and 6-carboxy-4-nitroquinoline 1-oxide, both active, and 4-nitroquinoline, which is inactive, were markedly inhibitory to the incorporation, while quinoline 1-oxide (inactive) showed no such inhibition. A tentative conclusion was reached that the inhibition of P^{32} incorporation into nucleic acid by this class of compounds may depend upon the presence of nitro radical at the position 4, and may not be related to their anti-cancer action.

The authors wish to express their appreciation to Dr. Waro Nakahara, Director of Cancer Institute, for his continued support and help in this investigation.

References

1. Sakai, S., Minoda. K., Saito, G., Akagi, S., Ueno, A., Fukuoka. F.: Gann, **46**, 605 (1955)
2. Fukuoka, F., Sugimura, T., and Sakai, S.: Gann, **48**, 65 (1957)
3. Lawrance, P. B., and Carter, C. E.: J. Cell & Comp. Physiol., **85**, 385 (1950)
4. Skipper, H. E. Mitchell, J. H., and Bennett, L. L.: Cancer Research, **10**, 510 (1950)
5. Skipper, H. E., Mitchell, J. H., Bennett, L. L., Newton, M. A., Simpson, L., and Eidson, M.: Cancer Research, **11**, 145 (1951)
6. Goldthwatt, D. A., and Bendich, A.: J. Biol. Chem., **196**, 841 (1952)
7. Cario, P.-E., and Mandel, H. G.: J. Biol. Chem., **201**, 343 (1953)
8. Davidson, J. D., and Freeman, B. B.; Cancer Research, **15**, 31 (1955)
9. Amano, M., Kihara, H. K., and Sibatani, A.: Bioch. Biophys. Acta, In Press.
10. Fukuoka, F., Sugimura, T., and Suzuki, S.: Gann, 253 (1957)
11. Swift, H.H.: Physiol. Zoöl., **23**, 169 (1950)

要　旨

In vitro における P^{32} の核酸へのとりこみに及ぼすキノリン誘導体の影響

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抗腫瘍性を有する 4-nitroquinoline 1-oxide ならびに 6-carboxy-4-nitroquinoline-1-oxide，抗腫瘍性を持たざる quinoline 1-oxide，及び 4-nitroquinoline による，P^{32} の核酸へのとりこみに及ぼす影響を Ehrlich 癌細胞を用い，in vitro でしらべた。

結果は抗腫瘍性を有する前物質は等しく P^{32} 取込みに対しても顕著な抑制を示し，抗腫瘍性を持たざる後物質中の 4-nitroquinoline は前 2 物質と同じく P^{32} 取込みに対して抑制を示したが，quinoline 1-oxid は全く抑制しなかった。

すなわち抗腫瘍の場合は，4 の位置に nitro 基を有し，さらに oxide 型であることを必要とするが，P^{32} 核酸とりこみの場合は，nito 基のみを必要とするのではなかろうかと推察せられる結果となった。

(文部省科学研究費による)

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BIOCHEMICAL INTERACTION OF TRYPAN BLUE AND p-DIMETHYLAMINOAZOBENZENE IN THE LIVER OF RAT (II): THE INHIBITORY EFFECT OF TRYPAN BLUE ON THE EXPERIMENTAL PRODUCTION OF LIVER CARCINOMA

(Plates XX and XXI)

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Iwase and Fujita (1953, 1954, 1955) found that subcutaneous injections of trypan blue (Merck) administered to rats receiving p-dimethylaminoazobenzene (DAB) inhibited liver tumour formation. Sayama, Miyaji, Taki, Uemura, Azuma, Takashi and Hachisuka using a different brand of the dye reported a similar result.

Miller and Miller (1947, 1953) showed that livers of rats fed with DAB contain aminoazo dyes bound by chemical linkage to certain liver proteins and that the formation of protein-bound dyes may be decreased or delayed by riboflavin and 20-methyl-cholanthrene which inhibit tumour formation. Trypan blue is a sulphonated dis-azo dye that somewhat resembles DAB in chemical structure and it seemed of interest to attempt to find out whether the inhibition was due to competition between the two substances for binding with the available protein. In early experiments regarding this problem, the majority of animals died, mainly from respiratory infection, and hence, the effects of trypan blue on the protein-bound DAB could be observed for only up to 6 weeks after the commencement of the experiments (Fujita *et al.*, 1956).

Further, the results obtained differed from those of Sayama et al. who found that trypan blue injections lowered the contents of protein-bound DAB of the livers.

It was therefore considered that more prolonged experiments with a larger number of experimental animals are necessary for a definite conclusion to be made.

It has been pointed out that the Merck product, and other commercial brands of trypan blue contain a variety of impurities. Our previous results on paper chromatography, adsorpto-chromatography on aluminium oxide and paper electro-

phoresis showed that the Merck preparation is a mixture of at least three components, red, purple and blue, and an attempt was made to determine which component was the most effective inhibiting agent.

Material and Methods

Rats. The rats were bred in our laboratory and housed in screen bottom cages in groups of 4 to 6. They weighed 80-130g. (3-4 months old) at the beginning of the experiments and were kept on a basal diet of unpolished rice containing 1.33-2.71mg. of riboflavin per kg. and greenstuff daily. To 1000g. of this basal diet, 10, 20, 30 and 45 ml. of a 2 per cent solution of DAB dissolved in olive oil were mixed (0.02, 0.04, 0.06 and 0.09 per cent DAB-containing diets respectively).

Trypan blue. Five g. of the "Trypan blue for injection" (Merck) were placed in 200ml. of ethyl alcohol and heated for about 30 minutes in a water bath at 60°C. After immediate filtration the precipitate was washed twice with a 20ml. portion of cold ethyl alcohol. The wet precipitate, wrapped in quantitative filter paper, was dried into powder at low temperature *in vacuo*. This was injected at bi-weekly intervals subcutaneously in doses of 1ml. of a 1 per cent solution in cold distilled water, into rats of the TB-DAB and TB groups described below.

Estimation of the total protein-bound DAB. The animals under ether anaesthesia had their abdomens opened, and the livers were perfused *in situ* through the vena cava with 50ml. of a 2 per cent sodium citrate solution. From one piece of the excised livers, 20 per cent homogenates were prepared using a Potter-Elvehjem homogeniser and employed for the preparation of the crude protein precipitates of Miller and Miller (1947). The dry powders obtained by this procedure represented 15.6 to 25.6 per cent (av.=20.1) by weight of the original fresh liver. For the estimation of protein-bound DAB, the method described by Miller and Miller was followed routinely.

Preparation of solutions of dye components. A quantity of 20ml. of a 1 per cent aqueous solution of trypan blue (Merck) was adsorbed on a 3×15cm. column of aluminium oxide (Brockmann). The dye first eluted by water is red (trypan blue r), while the dye next eluted is purple (trypan blue p). The dye completely eluted by 0.1M phosphate buffer is blue (trypan blue b). Each of the dye solutions thus obtained was adjusted to the original volume for injection and then used for the experiments of Series II. These dye solutions were freshly prepared for each injection.

By a similar manipulation, but employing only distilled water as developer, trypan blue b solution was separated from 500ml. of a 1 per cent trypan blue solution (the first solution of trypan blue b). The portions which were regarded to be a mixture of trypan blue p and b, were separated by passage again through

another series of aluminium oxide column into two dye solutions (the second solution of trypan blue b). The first and second trypan blue b solutions were combined and subjected to similar chromatography. Special attention was paid to the removal of red and purple components rather than the recovery of each dye contained in the branded dye, in the course of the above procedure. The portion of trypan blue b thus obtained was dried to light powders at low temperature *in vacuo*. It showed the appearance of clear blue cotton linter and of weight 1.94g. This was employed for injections of Series III as a 1 per cent solution in cold distilled water.

Experimental

Series I. A group of 50 rats was fed on the 0.06 per cent DAB diet, and given injections of trypan blue (TB-DAB group). A second group of 50 rats was given the basal diet without DAB and given trypan blue (TB group) and a third group of 50 rats the 0.06 per cent DAB with no trypan blue (DAB group). The feed and injections were continued until the animals either died or were killed at the end of the experimental periods of 2, 4, 6, 8, 10, 15 and 20 weeks. The rats were killed in groups of 3 to 10 at the end of each period.

Series II. A series of 68 animals was fed 0.06 per cent DAB diets for 2 months and thereafter 0.09 per cent DAB diet until a dose of 600–860 mg. had been ingested by each rat. The rats were divided into 4 groups of 15–20, the rats in 3 groups receiving 10 fortnightly injections of one of the dye components (TB-r, TB-p and TB-b groups respectively), simultaneously with the commencement of the feeding and 1 control group. The rats were allowed to die naturally or killed when very ill.

Series III. 30 rats were fed for the first month with a diet containing 0.02 per cent DAB, during the second month with one containing 0.04 per cent DAB, and thereafter with a diet containing 0.06 per cent DAB, till the amount of DAB taken in per rat totalled 500mg. After this a basal diet was given until the end of experiment. The rats were divided into 2 groups of 15, and to one group, a 1 per cent solution of purified trypan blue b was injected as in the case of Series II, once biweekly, 10 times (trypan blue b group). On the 200th day after commencement of the experiment the animals were sacrificed.

Results

A previous report showed that the Merck preparation of trypan blue is a mixture of at least three varieties of dye, red, purple and blue components, and these in the absorption spectrum presented slight differences in the wave lengths of their maximum absorption for their respective visible regions. Of these dye components, the red portion appears to be most responsible for the reported toxicity of com-

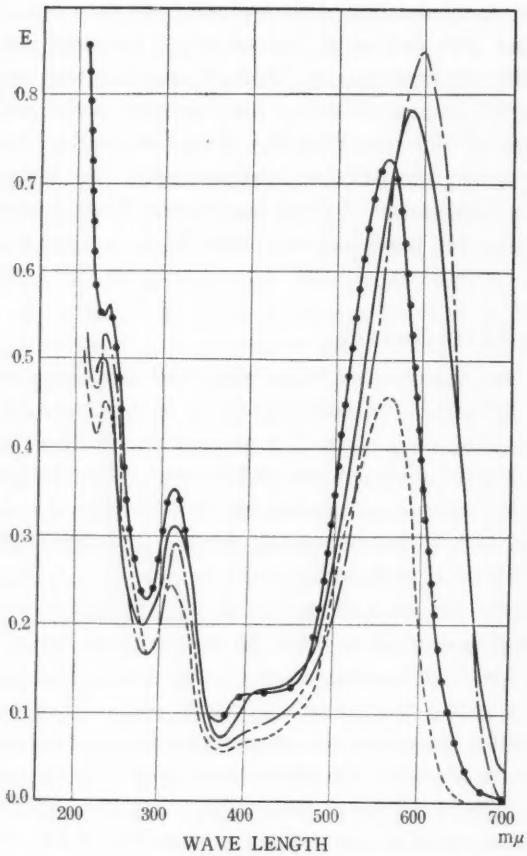

Fig. 1. Absorption spectrum of the alcohol-soluble component of the Merck's trypan blue.
———— : 0.005 per cent aqueous solution of trypan blue.
—•—•— : 0.00008 per cent aqueous solution of the alcohol-soluble dye.
-------- : Trypan blue r solution.
— - — : Trypan blue b solution. (Fujita et al. 1956)
Effective cell thickness=1.09cm.

mercial products, from the results of Series II experiment described below. In succeeding experiments with the Merck brand regarding protein-bound DAB, 64 out of 90 rats died within 6 weeks. Hence, an experiment was next undertaken with the preparations containing relatively small quantities of the red component. Trypan blue p and b are not soluble in 95 per cent ethyl alcohol but the red coloured bye-products believed to be produced during synthesis are soluble. As may be seen from Fig. 1, the alcohol-soluble constituent of the Merck brand absorbed maximally at 560mμ. in the visible region, and the shape of the absorption spectrum practically coincides with that of the red dye isolated by chromatography on aluminium oxide column. Absorption spectra of trypan blue r, trypan blue b and Merck brand are given in the figure for comparison. Despite the manipulation taken to remove the red dye portion, many of the animals stained with the dye solution (dye I) were in poor condition. The materials for the preparation of the crude liver precipitates were therefore derived from the 3 to 10 animals of each group in which the general appearance was comparatively good at the end of each experimental period.

Table 1 summarizes the results on the amounts of total protein-bound DAB in

Table 1. — The Estimation of Bound DAB at Periods of 2, 4, 6, 8, 10, 15 and 20 Weeks after Commencement of DAB Feeding.

	DAB Group		TB-DAB Group		TB Group	
	Rat No.	E Values	Rat No.	E Values	Rat No.	E Values
After 2 Weeks	6	0.196	11	0.126	1	0.035
	7	0.134	12	0.163	2	0.054
	8	0.160	13	0.144	3	0.030
	9	0.130	14	0.170	4	0.048
	10	0.150	15	0.158	5	0.068
Average for Group		0.154		0.152		0.047
After 4 Weeks	21	0.108	31	0.162		
	22	0.106	32	0.180		
	23	0.152	33	0.220		
	24	0.142	34	0.118		
	25	0.148	35	0.286		
	26	0.130	36	0.220	16	0.047
	27	0.166	37	0.188	17	0.048
	28	0.214	38	0.150	18	0.052
	29	0.166	39	0.172	19	0.062
	30	0.170	40	0.188	20	0.058
Average for Group		0.150		0.188		0.053
After 6 Weeks	46	0.146	51	0.192	41	0.056
	47	0.188	52	0.150	42	0.058
	48	0.272	53	0.188	43	0.062
	49	0.150	54	0.261	44	0.064
	50	0.158	55	0.168	45	0.053
Average for Group		0.183		0.192		0.059
After 8 Weeks	59	0.104	62	0.156	56	0.055
	60	0.150	63	0.160	57	0.078
	61	0.116	64	0.226	58	0.030
Average for Group		0.123		0.181		0.054
After 10 Weeks	68	0.096	71	0.072	65	0.064
	69	0.096	72	0.148	66	0.078
	70	0.112	73	0.086	67	0.068
Average for Group		0.102		0.102		0.070
After 15 Weeks	77	0.096	80	0.094	74	0.070
	78	0.086	81	-rat died-	75	0.048
	79	0.096	82	-rat died-	76	0.068
Average for Group		0.093		0.094		0.062
After 20 Weeks	86	0.118	89	0.112	83	-rat died-
	87	0.114	90	0.088	84	-rat died-
	88	-rat died-	91	-rat died-	85	-rat died-
Average for Group		0.116		0.100		

the livers of the TB-DAB, DAB groups. All values of the estimation were expressed as E $(\log_{10} {}^{I_0}/_{I})_{520m\mu}$ per 100mg. of the dry powder under the conditions described by Miller and Miller. The values obtained from the rats of the TB group coincided with that (0.050) reported by Miller et al. for rat liver fed on a basal diet. As shown in the Table, throughout the total experimental periods of

DAB feeding the amounts of protein-bound DAB in the livers of the TB-DAB group were either approximately equal to or slightly higher or lower than that of livers in the DAB group. Furthermore, as may be noted from the data at the end of 4 weeks, the individual differences in each group were fairly large, there being noted even a higher average for the TB-DAB group than for the DAB

Table 2. — The Inhibiting Effects of Trypan blue r, Trypan blue p. and Trypan blue b on the Experimental Production of Liver Cancer.

Dye injected	No. of days fed	Amounts of DAB ingested (mg.)	No. of animals		Liver findings**					Incidence of liver cancer, (per cent)
			at initial	at final	—	±	+	++	+++	
Trypan blue R.	130—146	600—729	20	12	0	0	0	0	12	100
Trypan blue P.	120—125	615—629	15	14	0	0	5	4	5	100
Trypan blue B.	109—126	629	7	7	2	2	3	0	0	43
Trypan blue B.	128	861	10	8	0	0	8	0	0	100
Control	140—147	628	16	15	0	0	0	0	15	100

Dye injected	No. of animals	Liver findings at autopsy (m, male; f, female)								
Trypan blue R.	20	m, — 7	m, — 21	m, — 21	m, — 32	f, — 42	m, — 57	f, ± 66	f, ++ 91	f, +++ 130
		m, +++ 130	f, +++ 130	f, +++ 130	f, +++ 146	f, +++ 146	f, +++ 146	f, +++ 146	m, +++ 146	f, +++ 146
		f, +++ 146	m, +++ 146	days						
Trypan blue P.	15	f, ± 90	m, ++ 120	m, + 121	f, + 125	f, ++ 125	f, ++ 125	m, + 125	f, +++ 125	f, +++ 125
		f, ++ 125	f, +++ 125	m, +++ 125	f, + 125	f, + 125	f, +++ 125	days		
Trypan blue B.	7	f, — 109	f, + 126	f, — 126	m, ± 126	f, + 126	m, ± 126	f, + 126	days	
Trypan blue B.	10	f, ‡ 23	m, ‡ 35	f, + 128	m, + 128	m, + 128	f, + 128	m, + 128	m, + 128	m, + 128
		m, + 128	days							
Control	16	m, — 66	f, +++ 140	f, +++ 140	f, +++ 140	f, +++ 140	f, +++ 140	m, +++ 140	m, +++ 140	m, +++ 140
		f, +++ 140	m, +++ 140	m, +++ 147	f, +++ 147	f, +++ 147	f, +++ 147	f, +++ 147	days	

‡ These rats were lost by cannibalismus.

** — indicates smooth, practically normal liver; ± indicates nodular cirrhosis with adenomatous hyperplasia; + indicates distinct areas of cholangioma or hepatoma or both; ++ indicates extensive liver cancer without metastasis; +++ indicates extensive liver cancer with metastasis (Sugiura, 1948).

group. Such results might suggest that on the basis of the method of Miller and Miller, the differences in the amounts of bound DAB between the TB-DAB and DAB groups are insignificant and the inhibitory effects of trypan blue cannot be explained on the basis of competition between the two azo dyes.

Next, experiments were made to find out which of three varieties of dye inhibits DAB-induced carcinogenesis. The above-mentioned red, purple and blue dyes on the adsorpto-chromatogram were separated as much as possible quantitatively from a 1 per cent aqueous solution of the Merck preparation, and injected into young adult rats of the TB-r, TB-p and TB-b groups during ingestion of DAB.

The majority of the rats in the TB-r, TB-p and control groups lost body weight from the beginning of the experiment, and thereafter maintained their weight for about 3 months. On the other hand, animals on the blue dye solution (dye II) maintained their body weights or gained during the first 3 months. Practically all animals presented a healthy appearance throughout the experimental period of 128 days. The mortality rate in the TB-b group was 6.3 per cent on the 100th day and those of the TB-p and control groups, 6.7 and 6.3 per cent respectively. But the mortality rate in the TB-r group was 40 per cent on the 100th day, and showed a higher value than the other three groups. All rats dying before the 70th day were free of tumours. Length of survival and tumour incidence are stated in Table 2. As may be seen from the data, no definite difference can be seen between the incidence of the liver cancer observed in the three experimental groups and the controls. However, in the rats of the TB-r and control groups which were given 600 to 729 mg. of DAB per rat, all 12 of the former, and all 15 of the latter autopsied during the 130 to 147 experimental days had developed extensive liver cancer with metastases (Plate XX, Fig. 1, Plate XXI, Fig. 4). Of 14 rats of the TB-p group, 9 showed extensive liver cancer, of which 5 cases were accompanied by metastases, and distinct areas of cholangioma or hepatoma or both were found in the remaining 5 (Plate XXI, Fig. 5). On the other hand, in rats of the TB-b group in which the average amount of DAB ingested was 629mg., there were no obvious lesions due to DAB in 2 cases and nodular cirrhosis with adenomatous hyperplasia was noted in 2 cases (Plate XX, Fig. 2). Further, although 8 cases in the TB-b group fed with 861mg. of DAB developed circumscribed liver cancer, they were in general smaller than those of the control, and were not associated with metastases (Plate XX, Fig. 3). Such results might suggest that the blue component was most closely related to the commercial trypan blue as regards its protection.

Although it is obvious that the above-mentioned blue dye contains much less the red and purple components, when compared with the Merck brand, it cannot be said that these bye-products do not exist at all. A similar biological experiment using a more pure form of trypan blue b (dye III) was thus undertaken.

In order to clarify the effect of this dye, the experiment was discontinued when the incidence of liver cancer observed in the control rats was regarded to be 50 to 70 per cent by palpation.

However, no inhibiting effect on the neoplasmic transformation was observed

Table 3. — The Inhibiting Effect of Purified Trypan blue b on the DAB-Carcinogenesis.

Rats receiving ingestion of DAB and no injection of trypan blue b over 151 days.					Rats receiving ingestion of DAB and 10 injection of 10 mg. trypan blue b over 147 days.				
Rat No.	Sex*	Survival in days	Amounts of DAB ingested (mg.,)	Liver findings	Rat No.	Sex*	Survival in days	Amounts of DAB ingested (mg.)	Liver findings
1	f	139	425	♯	1	m	2	—	—
2	m	181	500	++	2	m	193	500	♯
3	m	197	500	++	3	m	200	500	+
4	m	200	500	++	4	m	200	500	++
5	m	200	500	+++	5	m	200	500	+++
6	m	200	500	+++	6	m	200	500	+
7	m	200	500	+++	7	m	200	500	+++
8	m	200	500	++	8	m	200	500	++
9	m	200	500	++	9	m	200	505	++
10	m	200	500	—	10	m	200	500	++
11	m	200	500	—	11	m	200	500	±
12	m	200	500	+	12	m	200	500	±
13	m	200	500	+	13	m	200	500	—
14	m	200	500	—	14	f	200	500	+
15	m	200	500	—	15	f	200	500	—
Tumour incidence: 71.4% (on the 181—200 th days)					Tumour incidence: 69.2% (on the 200 th day)				

* Male was expressed as m. and female, f.

♯ These rats were lost by cannibalismus.

Liver findings at autopsy were described as in Table 2.

in the blue dye-treated rats. As may be seen in Table 3, the tumour incidence of the control group was 71.4 per cent, while that of the trypan blue b group was 69.2 per cent. Further, no differences at all in pathological features were noted between stained rats and controls. It was noted, in the course of this experiment, that the stained rats showed only a slight blue generalized coloration after each injection. Thus, the impression remained that the purer the blue dye the more likely for the vital staining potency to be weak. Again, no toxicity reported for the commercial brands of trypan blue was practically noted from the rats receiving 10 fortnightly injections of this blue component, as well as that of Series II.

Discussion

Sayama et al. (1954) claimed that the injections of trypan blue into rats resulted in a reduced amount of protein-bound DAB and they considered that this was associated with the lower incidence of liver cancer. Their conclusions are questionable, however, as the differences they report are hardly significant.

Our early observations at the ends of 2, 4 and 6 weeks after the beginning of DAB administration suggested that trypan blue produced practically no effect on total protein-bound DAB in the livers of rats fed with DAB. It has been reported by Miller and Miller (1947) that when rats are fed with DAB the amount of protein-bound dye in the liver reaches a maximum between the 4th and 6th weeks, followed by a gradual decrease later. The data herein presented indicated that the average values of bound dye in both groups reach each maximum after 6 weeks of feeding and thereafter slowly decreased. Further, throughout a dye-feeding period of 20 weeks there was no significant difference in value between trypan blue-stained rats and controls. But it must be pointed out that the values up to the 8th week show fairly large variations, depending on the individual rat.

The samples used for estimation of protein-bound DAB were crude liver precipitates. Even though no differences are seen when the crude preparations of Miller and Miller are used, a significant difference may be demonstrated when the amounts of DAB bound to some particular protein of the crude preparations are compared. From the present data therefore, one may suggest that trypan blue is not effective in reducing the absorption of DAB from the intestine or in increasing the rate of metabolism of DAB in the liver.

Recent studies on the biological actions of trypan blue showed that when the dye is injected subcutaneously into rats at weekly or biweekly intervals reticulum cell sarcoma of the liver may be induced (Gillman and Gillman, 1949; Simpson, 1952). Trypan blue which leads to the formation of sarcoma when injected into rats and yet has an inhibitory effect on DAB-induced carcinogenesis, therefore resembles 20-methyl-cholanthrene which inhibits liver tumor formation in rats receiving 3′-methyl-DAB (Richardson, Stier and Borsos-Nachtnebel, 1952; Miller and Miller, 1953). Merck's trypan blue is not a pure product (Colour index of Merck), and it is therefore uncertain whether the preparation used contains both carcinogen and anticarcinogen, or whether there is some agent that induces sarcoma but which inhibits DAB carcinogenesis.

It is evident from the results of Series II, that of the various dye components of the Merck preparation, the blue-coloured component on the aluminium oxide column most closely resembled in its protective effect the branded dye. In spite of the prolonged treatment by the blue component, however, the rats did not show

a clear-cut inhibition, when compared with that reported for the Merck and Gruebler products (Sayama et al., 1954; Iwase, 1954). Variations in the amount or kind of impurities present in the blue-coloured component compared with those in the Merck stain used in the previous work, might account for some of the differences. This is probably related to the differences in toxicity reported for various commercial products of trypan blue, as has been pointed out by Simpson (1952) and others. While the Merck-stained rats lost appetite and weight after each injection, those on the blue component produced no difference in the mortality rate from the control group. Further, removal of red and purple components from the Merck preparation resulted in a reduced amount of trypan blue particles taken up by the reticuloendothelial system, in the order of dye III, II and I. It may be supposed that there thus exists a certain interrelationship between the vital staining potency, and anticarcinogenic effects or toxicity of trypan blue, but the details are not yet clear.

Summary

The red-coloured component contained in the commercial brands of trypan blue is removed by treatment with hot ethyl alcohol.

By this manipulation, a dye containing relatively small quantities of the red dye component was prepared from the Merck brand to clarify whether the formation of protein-bound DAB in the livers is decreased or delayed in the rats receiving biweekly trypan blue and DAB, when compared with those on DAB alone.

Throughout a dye-feeding period of 20 weeks the bound DAB showed no significant difference in the amount and period of maximum level between trypan blue-stained rats and controls.

The red, purple and blue components contained in a solution of trypan blue (Merck) were separated by adsorpto-chromatography on aluminium oxide, and the inhibiting effects of each component on the DAB-carcinogenesis were determined. Of these dye components, the red and purple dyes did not delay the formation of hepatic neoplasia at all, in doses contained in a 1 per cent solution of the Merck preparation. The blue dye most closely resembled in its protective effect the branded dye, but the effect by this component was not a clear-cut inhibition, as has been shown to occur previously with the Merck brand.

It could not be stated that the above-mentioned blue dye contains no red and purple components. The blue component was repeatedly purified by a similar adsorpto-chromatography, in order to remove red and purple bye-products. Although the experiment was discontinued when the rats on DAB alone showed a tumour incidence of 71.4 per cent, no anticarcinogenic effect was demonstrated in those on this dye preparation and DAB.

In what kinds and in what amounts these bye-products of the commercial brand should be present in trypan blue solution in order to produce minimum toxicity and simultaneously exhibit powerful anticarcinogenic effects, are matters that still remain unclear.

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References

Fujita, K., Iwase, S., Matsubara, T., Ishiguro, I., Matsui, H., Mizuno, T., Arai, T., Takayanagi, T., Sugiyama, Y., and Shirafuji, K.—(1956) "GANN" 47, 181.

Gillman, J., and Gillman, T.—(1949) Clin. Proc., Cape Town, 8, 222.

Iwase, S., and Fujita, K.—(1953) Nagoya J. Med. Sci., 16, 307.

Iwase, S., and Fujita, K.—(1954) "GANN", 45, 383. (in Japanese).

Iwase, S.—(1954) Nagoya J. Med. Sci., 17, 464.

Iwase, S., and Fujita, K.—(1955) Nature, 175, 552.

Miller, E. C., and Miller, J. A.—(1947) Cancer Res., 7, 468.

Miller, J. A., and Miller, E. C.—(1953) 'Advances in Cancer Research', 1, 339. New York (Academic Press, Inc.).

Richardson, H. L., Stier, A. R., and Borsos-Nachtnebel, E.—(1952) Cancer Res., 12, 356.

Sayama, Y., Miyaji, T. Taki, I., Uemura, E., Azuma, S., Takashi, S., and Hachisuka, T.—(1954) "GANN", 45, 386.

Simpson, C. L.—(1952) Brit, J. exp. Path., 33, 524.

Sugiura, K.—(1948) Cancer Res., 8, 141.

Explanation of Plates (XX and XXI)

Fig. 1. Livers on 140th day, of rats fed with 628mg. of DAB per rat, showing typical extensive liver cancer. ×0.44

Fig. 2. Livers on 126th day, of rats fed with 629mg. of DAB and received 10 injections of trypan blue b per rat, showing smooth, practically normal, or nodular cirrhosis with adenomatous hyperplasia, or distinct areas of cholangioma and/or hepatoma. ×0.46

Fig. 3. Livers on 128th day, of rats fed with 861mg. of DAB and received 10 injections of trypan blue b per rat, showing circumscribed liver cancer. ×0.46

Fig. 4. Livers on 130th day, of rats fed with 600-729mg. of DAB and received 10 injections of trypan blue r per rat, showing typical extensive liver cancer. ×0.47

Fig. 5. Livers on 125th day, of rats fed with 615-629mg. DAB and received 10 injections of trypan blue p per rat, showing circumscribed and extensive liver cancer. ×0.47

要　旨

ラッテ肝におけるトリパン青とパラヂメチルアミノアゾベンゼンの生化学的相互作用 (II): 実験的肝癌発生に対するトリパン青の抑制効果

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トリパン青による DAB 肝癌抑制についての当面の問題は，2 点に集約されよう。第 1 は，トリパン青が Miller 等の肝蛋白結合 DAB に対してどのような影響を与えるのか。この点についての従来の報告には，結合 DAB 量に対して，リボフラビンまたは 20—メチルコラントレンと同じように，その量を減少させ，また maximum level の時期をおくらせるという報告と，このような作用を全く示さないという報告がある。第 2 は，市販のトリパン青には種々の不純物，または副生色素が含まれていて，メルク製品の場合，少くとも赤色，紫色及び青色の 3 種色素の混合物であった。このような副生色素が市販のトリパン青の肝癌抑制においてどのような作用をもつものか。本報告は，これら 2 つの問題に対する結果を取扱っている。

1)　トリパン青の市販製品に含まれる赤色色素は熱エタノールによって除くことができる。

2)　上の操作によってメルクのトリパン青から赤色色素を除いた色素標本が，1% 水溶液として 2 週間毎にラッテの皮下に注射された。20 週に亘る DAB 飼育期間を通じて，結合 DAB の量及び maximum level の時期には有意義な差を見出し得なかった。

3)　1% トリパン青水溶液を活性アルミナによる吸着クロマトグラフィーによって，赤色，紫色及び青色成分に分離する。これらの色素成分のうちでは，青色色素成分が最も肝癌抑制効果が強いけれども，もとの市販製品に比べると，その効果は弱い。しかし，毒性もまた，低下することが注目された。

4)　3) の場合のトリパン青の青色色素は，副生色素である赤色及び紫色色素がもとの製品より少いことは明らかであるが，それらを全く含まないものではない。市販のトリパン青中の青色色素が活性アルミナにより繰返し純化された。この色素標本を用いた場合，もはや肝癌抑制効果は完全に失われ，生体染色能力も毒性も著しく低下した。市販製品の中の副生色素のどのような種類のものが，どのような量に含まれるとき，最小の毒性で最大の肝癌抑制効果を示すか今なお明らかではない。

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[GANN, 48, 289~296; October, 1957]

CYTOCHEMICAL STUDIES ON TUMOR CELLS, II. LOCALIZATION OF PHOSPHOLIPIDS IN NORMAL AND NEOPLASTIC TISSUES, AS REVEALED BY THE ACID HEMATEIN TEST

(With Pltes XXII and XXIII)

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There no longer seems to be any doubt but that phospholipids play a very important role in cells as indispensable components. According to Pearce (1954) the compound lipids are divided into (a) ester phosphatides, (b) acetal phosphatides, and (c) cerebrosides. An ester phosphatide generally consists of four components, phosphoric acid, glycerol, fatty acid and another which is a base or amino acid, or something of the sort. Phosphatides are usually counted among lipids, because of their lipid character; they also possess a marked tendency toward hydrophily. For this reason, phosphatides represent compounds which are intermediate between hydrophobic and hydrophilic substances existing in the cytoplasm. (*cf.* Desnuelle 1952, Frey-Wyssling 1953).

Bensley (1937) reported that a mitochondrion was a mosaic consisting of protein, glyceride, phospholipid and cholesterol molecules. Bourne (1952) was in agreement with the above view. Claude (1946) and Chantrene (1947) obtained evidence suggesting that considerable amounts of phospholipids are contained in mitochondria and in microsomes. Further, it has been learned that phospholipids make mitochondria stainable supervitally with Janus green. From his biochemical studies with beef heart muscles, rabbit thigh muscles, pus cells and tumor cells, Stoneburg (1939) has ascertained that nuclei contain lipids, especially rich in content of phospholipids and cholesterols. As is well known, the nucleolus also contains phospholipids (Vincent 1955). Levin *et al.* studied phospholipid metabolism during hypertrophy and hyperplasia in rat prostates and seminal vesicles and learned that the mitotic activity was associated with a maximum P^{32} uptake in phospholipids. It may be of interest to know how phospholipids are distributed in normal and neoplastic cells, and what difference occurs in the phospholipid-distribution or content between normal and neoplastic cells.

The acid hematein test after Baker (1946) is based on the fact that hematein dissolved in an acid solution shows specific affinity to phospholipids. Rennels (1953) applied Baker's test to sections of the anterior pituitary gland of rats to differentiate acidophile cells from beta- and delta-basophile cells. The present study, with the application of the acid hematein test, was carried on as an inquiry into whether any cytochemical difference exists in the MTK-sarcoma III, the spindle-shaped cell sarcoma, the Yoshida sarcoma, as well as several kinds of normal tissues of adult and newborn rats.

Contribution No. 397 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

Material and Method

Normal tissues for study were derived from the stomach, duodenum, pancreas, kidney and liver of adult and newborn rats. Rat fibroblasts cultured *in vitro* also furnished material. The MTK-sarcoma III, the Yoshida sarcoma and the spindle-shaped cell sarcoma were chosen as neoplastic materials for study. The MTK-sarcoma III, a rat ascites tumor, at the 300th transfer generation supplied material. The spindle-shaped cell sarcoma which developed in an albino rat by subcutaneous injections with methylcholanthren was used as the tissue culture material.

The method of staining. For the application of Baker's acid hematein test for phospholipids, sections of the materials as mentioned above were made with the use of polyethylene glycol wax instead of cresol gelatin. The fixed material was washed in running water for 6 or more hours and placed first in wax 1500 for 1 hour, then in the mixture of wax 1500 and 4000 in equal amount, and finally in wax 4000 with two changes. Then the material was embedded in wax 4000 in a paper cup. The sections were cut 5 to 7μ thick; they were floated on a formal-calcium solution containing an appropriate amount of sugar. As an affixative medium the mixture of gelatin, distilled water, glycerine and phenol was used according to Pearce 1954.

The material such as fibroblasts cultured on coverslips and tumor ascites were washed after fixation in tap water for 24 hours and stored in formal-calcium until further mordanting with dichromate-calcium. The tumor ascites derived from tumor-bearing rats was fixed with formal-calcium in centrifuge tubes and the following procedure was carried out in the tube.

The culture method. Normal fibroblasts and spindle-shaped cell sarcoma were cultured subjected to the roller-tube method; tissue taken from animals under asceptic condition was cut into one cubic millimeter pieces and planted on coverslips in clots composed of equal parts of chicken plasma and 50% chick embryo extract in Gey's solution. Then, coverslips were put into tube and fed with 2ml culture medium consisting of one part of 50% chick embryo extract, one part of horse serum, three parts of Gey's solution, about 200 units of penicillin G and about 200μg of streptomycin. Tubes were then incubated in the rotating drum (15 rotations per hour) at 37°C. Flourishing cultures were employed for the test at various intervals after incubation.

Hepatectomy. The material for regenerating liver cells was obtained from three partially hepatectomized rats. The livers were removed for test 2 days after operation.

Injection experiment of the tumor ascites into the liver. The tumor ascites derived from the MTK-sarcoma III and the Yoshida sarcoma was injected with

glass pipettes into livers of rats. The livers were removed for test 4 days after operation.

Observations

Kidney. Cells from the proximal convoluted tubule contain a considerable amount of substance intensely stained blue-black in their cytoplasm (Fig. 1). The border of cells is unstained. Epithelial cells of the Henle's loop showed blue-black round or elongated particles which are distributed throughout the whole of the cytoplasm, though the particles are not abundant. It remains unknown whether they are mitochondria or not. A Golgi-like structure is observable in some of the epithelial cells (Fig. 2). There is no visible difference in reaction of test between the adult and new born tissues.

Pancreas. Adult: Secretion granules which distribute in the distal part of the cytoplasm of acinar cells are deeply stained with acid hematein. Mitochondrial filaments are well defined in the homogeneous, colorless basal ground of the cytoplasm (Fig. 3). Cells of the Langerhans' islet also contain a considerable amount of intensely stained granules (Fig. 4). It is uncertain whether the stainability is different or not in different types of cells. But, there is often evidence showing that cells of different types show different reactions to stain.

Newborn: Cells of the pancreas of newborn rats are different from those of adult ones in that the cytoplasm of acinar cells is wholly filled with numerous secretion droplets of large size. It is therefore difficult to detect the mitochondrial filaments in the cytoplasm.

Liver. The cytoplasm of parenchymal cells is rich in blue-black bodies which assume the form of rod and filament and distribute throughout the whole cytoplasm (Fig. 5). The parenchymal cells of hepatectomized rats show many vacuoles which are negative to the test. The narrow cytoplasmic areas surrounding vacuoles contain blue-black granules. The stainability with acid hematein was found similar in the liver cells of normal and of hepatectomized rats. Cells of the bile duct contain no granules which stained with acid hematein.

Stomach. Cells of the epithelium show in the cytoplasm a small, well stained area in the upper side of the nucleus. Except this area, the supranuclear area of cells appears transparent of faintly granular. The mucigen granules were not detectable in sections (Fig. 6).

The cytoplasm of parietal cells contains numerous short rod-shaped granules stained blue-black. The secretory canaliculi are visible, well-defined in the cytoplasm (Figs. 7 & 8). In contrast to parietal cells, the cytoplasm of zymogenic cells has less affinity to the dye. Secretory granules stain faintly gray in these cells.

Duodenum. The supranuclear region of the cytoplasm of columnar epithelial cells contains a number of filamentous mitochondria which are arranged longitudinally. The mitochondria of granular type aggregate on the underside of the nucleus. The two types of mitochondria are found stained with acid hematein (Fig. 9). The stainability of epithelial cells with acid hematein is generally different from cell to cell and is deeper in newborn tissues than in adult tissues.

The Paneth cells which are located in the bottom of Lieberkühn's glands contain large and round secretory granules; they are positive to the acid hematein test (Fig. 10).

Cultured fibroblasts. The cytoplasm of fibroblasts in the tissue culture material contains numerous granules deeply stained; they are evenly distributed in the cytoplasm.

Malignant tissues. Following the intraperitoneal inoculation of the MTK-sarcoma III, the tumor cells propagate freely in the abdominal cavity, whilst at the same time they actively invade viscerous tissues surrounding the cavity, especially the omentum, and form a solid tumor (Hori 1957). The sections of the solid tumor stained with acid hematein showed that the tumor cells were negative to the Baker test, since all the cellular components are colorless.

Usually the tumor cells of the MTK-sarcoma III do not invade the liver tissue. In order to obtain the tumor-invaded liver the following operation was made. The tumor ascites derived from a tumor-bearing rat was inserted into an arbitrarily chosen lobe of a new rat. The liver was removed four days after the tumor injection and observed microscopically. The observation revealed that the tumor cells had propagated in the liver tissue. The sections stained with acid hematein showed under a low power magnification that the regions filled with tumor cells stain pale yellow, while parenchymal cells stain blue-black. That feature renders easy the differentiation between normal and neoplastic tissue (Fig. 11). The explanation why the cytoplasm of the tumor cell shows no affinity to acid hematein rests on two possibilities: one is that the cytoplasm of tumor cells may contain a less amount of phospholipids than that of normal cells and the other is that phospholipids of the cytoplasm of tumor cells may be in such a state as not to respond easily to the test. Detailed discussion on this point will be offered later.

The acid hematein test was also applied to the solid tumor of the Yoshida sarcoma. Similar results were obtained to those with the MTK-sarcoma III; the tumor cells were negative to the test.

Spindle-shaped cell sarcoma in vitro. Two types of cells were found to occur in the spindle-shaped cell sarcoma cultured *in vitro* as regards the reaction to Baker's acid hematein test. The one is referred to as BH-p type in which both the cytoplasm and nucleoplasm are diffusely stained blue-black with acid hematein.

The other is BH-n type in which both the cytoplasm and nucleoplasm fail to stain as a whole, only nuclear hof being stained. There is no definite morphological difference between these two types of cells (Figs. 12 & 13).

The MTK-sarcoma III and the Yoshida sarcoma. Baker's acid hematein test enables the distinguishment also of two types of tumor cells, BH-p and BH-n types, in the MTK-sarcoma III, as well as in the Yoshida sarcoma (Fig. 14). The two types of cells are recognizable in both mitotic cells and degenerating cells. For instance there are two types in dividing cells; cells of one type show the cytoplasm diffusely stained blue-black, while in cells of the other type the cytoplasm is entirely colorless.

Except the BH-p type cells, all cells showed nucleoplasm which was negative to the acid hematein test. Most of nucleoli showed negative reaction. But they showed affinity to the dye following the procedure of extraction of pyridine (Fig. 15). In supplement, Ciaccio's Sudan III method and the Smith-Dietrich method for demonstration of lipids were applied to the MTK-sarcoma III, but the results were not identical with those of the acid hematein test.

Discussion

It is widely accepted that mitochondria contain phospholipids. According to Claude (1944, 1946), mitochondria and microsomes are both composed of three components, ribonucleic acids, protein and lipids, and the last one, lipids, occupies 27% in weight in the mitochondrial fraction and 40–50% in the microsomal fraction. His experiment indicated further that phospholipids occupy 75% of lipid fraction in microsomes. Notwithstanding the presence of lipids in mitochondria, any cytochemical methods for lipids other than the acid hematein test have failed to stain mitochondria and microsomes. Amongst the most interesting results which have so far been obtained by the acid hematein test is, therefore, stainability of mitochondria with acid hematein. According to Lison (1953), however, mitochondria can not be stained, in some cases, even with acid hematein. This is probably due to the extreme smallness or the less lipid-content of mitochondria.

In the present experiment blue-black staining substances were demonstrated in the cytoplasm of liver, cells, those lining the proximal convoluted tubules and the Henle's loop of the kidney, those of the intestinal epithelium, the parietal cells of the stomach, and cultured fibroblasts; most of them are probably mitochondria although their filamentous structure has not been well preserved. Strangeways and Canti (1927) reported that formalin deformed filamentous mitochondria into rows of spheres. Baker (1955) thought that this was probably caused by the fact that those workers did not add an indifferent salt (calcium chloride) to the fixative. In this study, filamentous structure of mitochondria has not been well pre-

served in most cells examined, except for the acinar cell of the pancreas, in which filamentous mitochondria have fairly well been demonstrated. In addition to mitochondria, secretory granules were also stained with acid hematein in Paneth cells of the crypt of Lieberkühn and acinar cells of the pancreas. Evidence suggests a close relationship existing between mitochondria and secretory granules in connection with the lipid metabolism. It was shown by this study that most normal cells here investigated contained a varying amount of acid hematein-positive materials in their cytoplasm, while such materials could not be demonstrated in the neoplastic cells. Sections of the liver inoculated with the tumor have shown very clearly the difference of reaction to the test between normal and neoplastic cells. Electron microscope studies have generally indicated no significant difference in the cytoplasmic components between the normal and neoplastic cells (Dalton and Felix 1956, Selby *et al.* 1956). Albert and Johnson (1954) have reported that the phospholipid-content in the particle-fration of liver tumors was considerably low as compared with that of normal liver. Working on the analysis of lipid fractions of hepatoma, Kishi *et al.* (1939) have also found evidence that the iodine number of phosphatide in hepatoma was about 1/2 that of normal liver. These data seem to explain the present results wherein the acid hematein test has failed to stain the cytoplasmic particles of tumor cells. However, Albert and Johnson (1954) have also reported that both the primary induced and the transplanted liver tumors contained particle-nitrogen in much less quantity than normal liver. Low phospholipid content of liver tumors, therefore, would not indicate any significant alteration in the phospholipid concentration within the particulates. Cornatzer *et al.* (1953) expressed a different view: they have measured the phospholipid P and protein-bound P synthesis in the rabbit papilloma and skin with the aid of radioactive phosphorus, and found that there is a significant increase in those two substances in the papilloma when compared to normal skin. The results from the present study suggest that there seems to be a significant difference in the physicochemical nature of the phospholipids within cytoplasmic particles, probably within mitochondria, between tumor and normal cells. This difference may represent either the physical change of lipids from the unmasked state to the masked state, or perhaps the decrease of lipid content in tumor cells. The latter consideration seems to be more probable than the former.

The reason why two types of tumor cells exist only in the ascitic phase, but not in the solid phase of tumor, remains unknown. In the cytochemical studies of ten ascites tumors, Love *et al.* (1956) reported that all cells examined were negative to the acid hematein test. A re-examination of this point is now in progress by the present author.

Summary

The acid hematein test was applied to the stomach, duodenum, pancreas, kidney, liver and rat fibroblasts in culture, and to the MTK-sarcoma III, the Yoshida sarcoma and the spindle-shaped cell sarcoma. It was found that most normal cells here investigated contained varying amounts of the acid hematein-positive materials in their cytoplasm, while such materials have failed to be demonstrated in solid tumors so far examined. This seems to indicate a significant, physicochemical difference between tumor cells and normal cells with regard to phospholipids within cytoplasmic particles.

Two types of tumor cells, which showed a differential reaction to the acid hematein test, were distinguishable in the MTK-sarcoma III, the Yoshida sarcoma, and the spindle-shaped cell sarcoma in culture.

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Literature

Albert, S. and R. M. Johnson 1954. Cancer Res. 14: 271-276.
Baker, J. R. 1946. Quart. J. Micr. Sci. 87: 467-478.
———— 1955. Cytological technique. Methuen & Co. Ltd., London.
Bensley, R. R. 1937. Anat. Rec. 69: 341-353.
Bourne, G. H. 1952. Cytology and cell physiology. Oxford, London.
Chantrene, H. 1947. Biochim. et Biophys. Acta. 1: 437-448.
Claude, A. 1944. J. Exp. Med. 80: 19-29.
———— 1946. J. Exp. Med. 84: 51,61.
Cornatzer, W. E., D. G. Gallo, J. P. Davidson and R. G. Fisher 1953. Cancer Res. 13: 795.
Dalton, A. J. and M. D. Felix 1956. Ann. N. Y. Acad. Sci. 63: 1117-1140.
Desnuelle, P. 1952. Progress in the chemistry of fats and other lipids. Vol. 1. Pergamon Ltd. London.
Frey-Wyssling, A. 1953. Submicroscopic morphology of protoplasm. Elsevier Publ. Co. N.Y.
Hori, S. H. 1956. J. Fac. Sci. Hokkaido Univ. Ser. 6, Zool. 12: 480-486.
Kishi, S., T. Fujiwara, and W. Nakahara 1939. Gann 33: 332-341.
Levin, E., S. Albert, and R. M. Johnson 1955. Arch. Biochem. & Biophys. 56: 59-65.
Lison, L. 1953. Histochemie et cytochemie animales. Gauthier-Villars, Paris.
Love, R., E. V. Orsi, and H. Koprowski 1956. J. Biophysic. & Biochem. Cytol. 2: 1-14.
Pearce, A. G. E. 1954. Histochemistry. J. & A. Churchill Ltd. London.
Rennels, E. G. 1953. Anat. Rec. 115: 659-670.
Selby, C. C., J. J. Biesele and C. E. Grey 1956. Ann. N. Y. Acad. Sci. 63: 748-767.
Stoneburg, C. A. 1939. J. Biol. Chem. 129: 189-196.
Strangeways, T. S. P. and R. G. Canti 1927. Quart. J. Micr. Sci. 71: 1.
Cited from "Cytological technique" by J. R. Baker, 1955.
Vincent, W. S. 1955. "Structure and chemistry of nucleoli" in Int. Rev. Cytol. IV. Academic Press Inc. Publ., N. Y.

EXPLANATION OF FIGURES (Plates XXII and XXIII)

Reaction to the acid hematein test. For details see text.

Fig. 1. The glomerulus and convoluted tubules of the kidney. ×400.

Fig. 2. The Henle's loop. ×2000. Fig. 3. An acinar tubule of the pancreas. ×2000.

Fig. 4. Langerhans' islet of the pancreas. ×1000.

Fig. 5. Parenchymal cells of the liver. ×1000. Fig. 6. Epithelium of the stomach. ×1000.

Fig. 7. Parietal cells. ×1000. Fig. 8. The Gastric gland. ×100.

Fig. 9. The intestinal epithelium. ×1000.

Fig. 10. Paneth cells of the crypt of Lieberkühn. ×1000.

Fig. 11. The MTK-sarcoma III inoculated in the liver. The tumor cells are negative and liver cells positive to the test. ×400.

Figs. 12, 13. The spindle-shaped cell sarcoma *in vitro*. The cytoplasm of the BH-p type cell is black, while that of the BH-n type cell is colorless, except cytoplasmic granules aggregated in the nuclear hof. ×400.

Fig. 14. The MTK-sarcoma III in the rat ascites. ×800.

Fig. 15. Pyridine extraction test in the regenerating liver. The cytoplasm is negative, while the nuclear components are positive to the test. ×400.

要　旨

腫瘍細胞の細胞化学的研究 II.

酸ヘマテイン法による正常及び腫瘍組織の燐脂質の分布に関する研究

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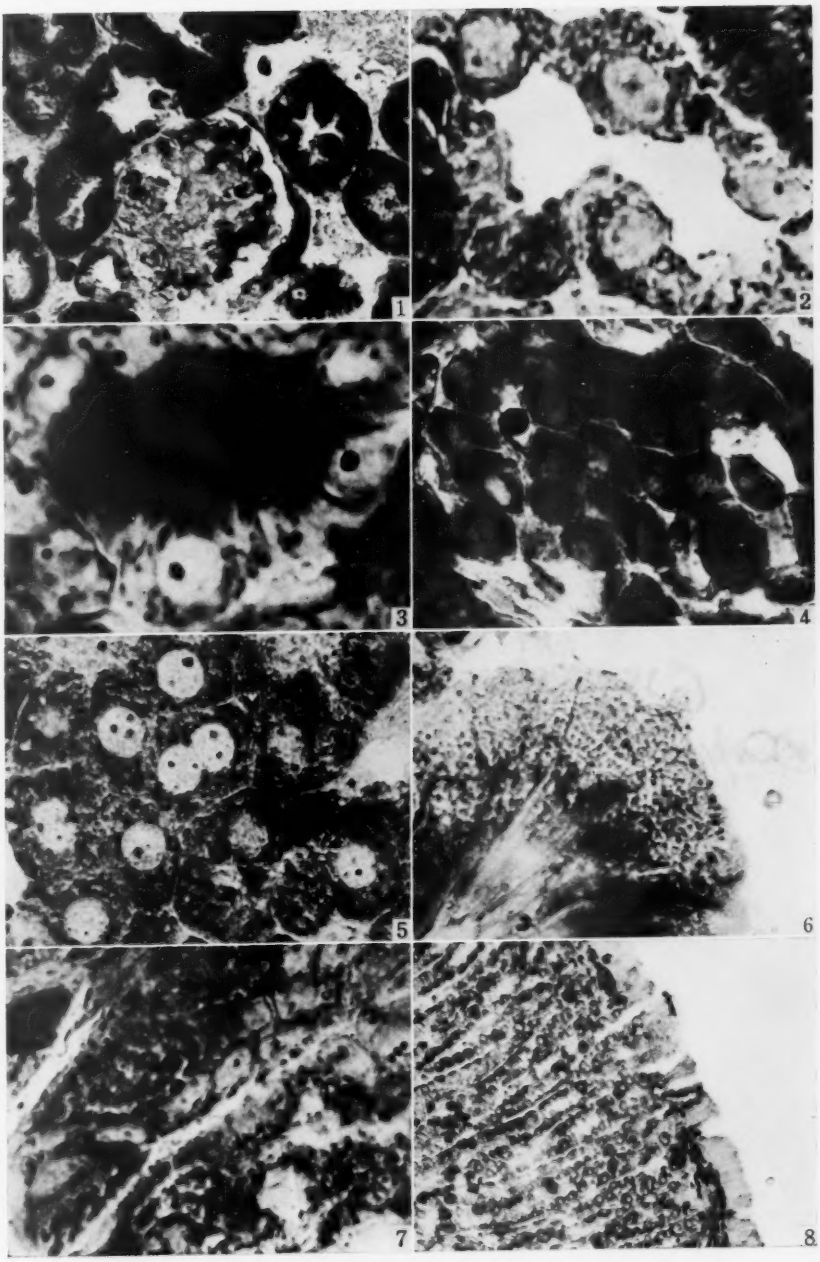
シロネズミの胃，十二指腸，膵，腎，肝の各正常組織，及び，MTK 肉腫 III，吉田肉腫，紡錘形細胞肉腫の腫瘍組織に酸ヘマテイン染色を施し，燐脂質の分布を比較調査した。その結果，正常組織細胞の大部分のものは，酸ヘマテインに対して陽性に反応する顆粒（多くの場合糸粒体と分泌顆粒）をその細胞質中に有しているが，腫瘍細胞は全く陽性物質を有さない。このことは肝へ腫瘍細胞を注入して行った実験において明瞭に示された (図 11 参照)。これらの事実から，腫瘍細胞の細胞質顆粒（主に糸粒体）は，正常細胞のそれに比して燐脂質の含有量が極めて少いのではないかと思われる。

次に，MTK 肉腫 III，吉田肉腫の腹水中に浮遊している細胞，及び組織培養した紡錘形細胞肉腫細胞を切片とせずに，酸ヘマテイン法で染色すると，その細胞全体が強い陽性反応を呈して青黒色に染るものと，全く反応が陰性で黄色を呈するものの 2 種類を区別することができた。すなわち，これら腫瘍細胞には燐脂質含有量の多い細胞と，非常に少い細胞があるのではないかと思われる。著者は前者を BH-p 型細胞，後者を BH-n 型細胞と名づけた。なお BH-p 型細胞の意義については目下調査中である。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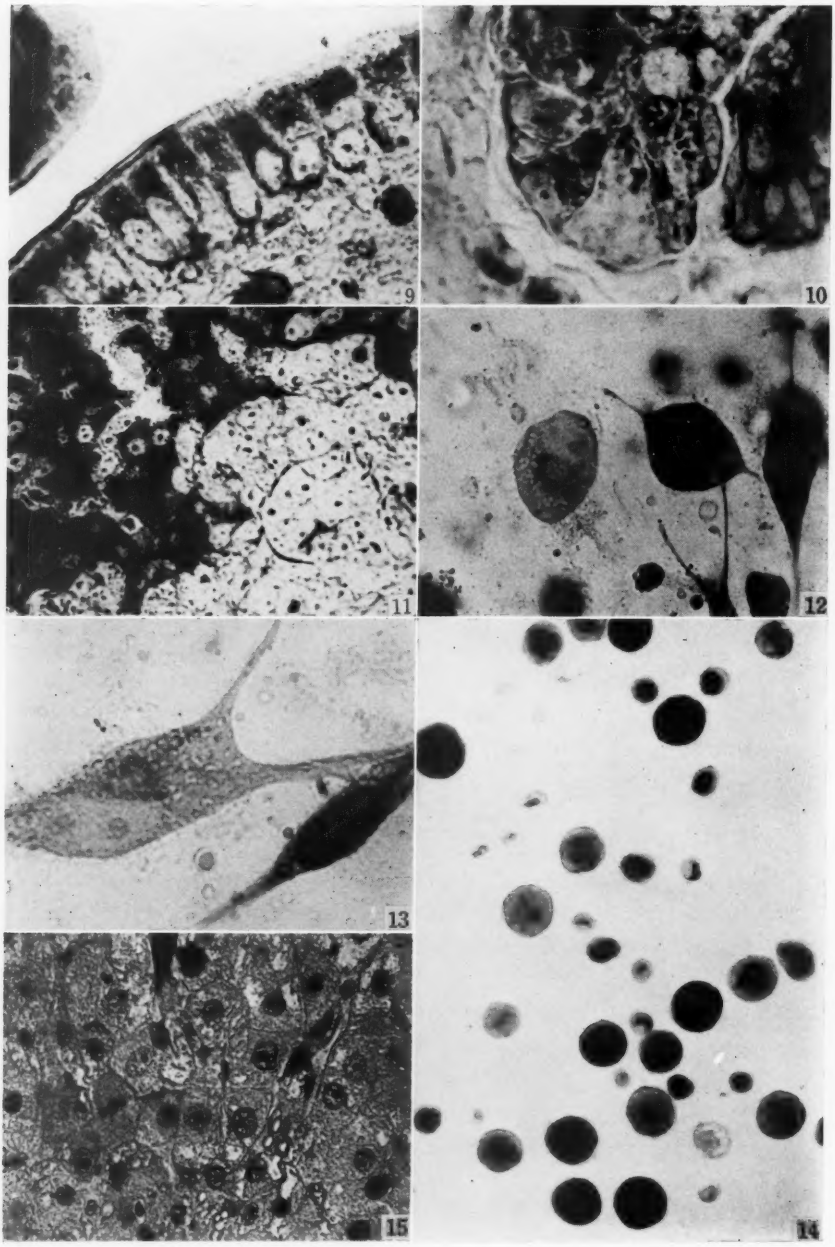
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[Gann 48, 297~304; October, 1957]

CYTOCHEMICAL STUDIES ON TUMOR CELLS, III. TWO TYPES OF TUMOR CELLS AS REVEALED BY THE ACID HEMATEIN TEST

(With Plate XXIV)

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In the course of cytochemical studies of phospholipids in certain ascites sarcomas and a spindle-shaped cell sarcoma, the present author has found that certain tumor cells show a selective reaction to the acid hematein test and that there are distinguishable by means of this test two types of cells in tumors, referred to as BH-p type cells and BH-n type cells. The former are positive to the test, stained deep blue, while the latter are negative (Hori 1957). The color distinction between the two types is very striking. The distinction of cell types was possible merely in an ascitic phase or *in vitro*, but not in a solid phase at all. The present study was undertaken with a hope to make clear the significance of the occurrence of two cell types in the tumor of an ascites phase alone but not in a solid phase.

Material and method: The MTK-sarcoma III and the culture material, the spindle-shaped cell sarcoma which was induced by the subcutaneous administration of methylcholanthrene in rats, furnished neoplastic samples for this study. The procedures of staining and culture follow those described in one of the author's previous studies (Hori 1957), except that explants of the spindle-shaped cell sarcoma were taken from the stock cultcre MS 3, not from tumor animals.

Observations

When the acid hematein test was applied to the formal-fixed preparations of the MTK-sarcoma III, BH-p type tumor cells were selectively colored as a whole: both the cytoplasmic ground substance and the nucleoplasm were stained deep blue. This predominant positive reaction to the test seems to be due to the existence of phospholipids, since blue coloration was not obtained in preparations of the same material which had been subjected to the pyridine extraction test. In addition to the BH-p type tumor cells, lymphocytes, monocytes and erythrocytes were usually colored with acid hematein. The tumor ascites contains a number of mononucleate leucocytes which are stained blue with this stain, and therefore

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it is a matter of difficulty to distinguish accurately the BH-p type tumor cells from the leucocytes because of the similar staining reaction. Mast cells do not react to the test. Further, there are observable BH-n type cells in the tumor ascites subjected to the test; their cytoplasm as well as nucleoplasm are wholly negative to acid hematein, being yellow as a whole. Moreover, there are present tumor cells which are transitional from BH-p type to BH-n type; their cytoplasm stains blue and the nucleoplasm yellow with acid hematein. Further, there is a group of cells in which the cytoplasm and nucleoplasm show dirty brown color after the application of the test; they are designated as BH-n type in the following description.

The tumor material was fixed with formal-calcium in a centrifuge tube with which the acid hematein test was applied. The tumor cells in the fixed material retained their spherical form; they were rather indistinct in cytoplasmic structure. Consequently, the stainability of the cytoplasm with acid hematein in relation to various mitotic phases was not clear. There were present certain telophase cells in which the cytoplasm reacted positively to the test, being blue in color, while some others which showed negative reaction. Observations of tumor cells in the culture material (MS 3) have given further detailed information on the internal structure of cells stained with acid hematein, as follows:

The cytoplasm of BH-p type cells was diffusedly colored blue as a whole *in vitro*. Their nuclei as well as nucleoli showed a similar staining reaction. Usually mitochondria were not observable at all. Vacuoles remained unstained after the application of acid hematein test, while they showed positive pseudoplasmal reaction (Hack 1952) to Schiff's reagent. On the other hand, the cytoplasm of BH-n type cells generally showed *in vitro* a negative reaction to the test. But the nuclear hof and/or cortical area of the nucleus often showed a faintly blue coloration with a number of minute granules well-stained. Both nuclei and nucleoli were negative to this test.

From the above results it is evident that the BH-p type cells contain much phospholipids than the BH-n type cells. Further, the frequency of such cells was obtained by calculating the number of the BH-p type cells per total number of tumor cells in the ascites; it was found to range from about 60% to 30% in general, but sometimes a 0% frequency occurred. There was no special correlation between the mitotic frequency and the frequency of the BH-p type cells in the tumor ascites.

The effect of pH of the fixative on the stainability of BH-p type cells with acid hematein was observed as follows:

1) The fixative was prepared with 90ml. of Tyrode's solution, 10ml. of formalin and 1 g of calcium chloride anhydrate, and the pH was adjusted to 6.5, 7.0, 7.6 in

three bottles by adding 1/10N HCl. The tumor ascites was fixed with these fixatives and the results were compared in relation to the frequency of the BH-p type cells in the ascites. There was found no appreciable difference in frequency of the BH-p type cells in the present experiment.

2) Six kinds of fixatives were prepared. Three were the same as those used in the previous experiment. The remaining three consisted of 9 parts of Tyrode's solution and 1 part of formalin containing no calcium chloride; their pH values were adjusted to 6.5, 7.0, 7.6 in three bottles, respectively. The tumor ascites was fixed in these fixatives, and subjected to the acid hematein test. Again, the results were compared in respect to the percentage of BH-p type cells. There were found few cells of that type in the preparations which were fixed with fixatives without calcium chloride. The different pH values of the fixative exerted no influence upon the appearance of BH-p type cells.

From the above results the conclusion may be possible that a selective demonstration of the BH-p type cell needs the presence of $CaCl_2$ in the fixative.

Brief chromation for 1 hour at 60°C carried out prior to staining with acid hematein was also an indispensable procedure in order to obtain a selective demonstration of BH-p type cells.

Though the explicit existence of BH-p type cells was proved in the ascitic phase of the tumor, there were recognizable no such cells in sections of the solid tumor. This was the case not only in the MTK-sarcoma III, but also in the Yoshida sarcoma (Hori 1957). In the case of the methylcholanthrene-induced tumor, they were found in tissue culture preparations, but not in tissue sections. In order to answer the question why the phospholipid-containing cells have not been observed in the solid tumor, the following experiments were undertaken using the MTK-sarcoma III as material.

3) The solid tumor which was produced through tumor infiltration into the *omentum* was removed from an animal, and homogenated by means of a glass homogenizer. The resulting tumor cell suspension was fixed with formal-calcium and subjected to the test.

4) Tumor ascites was enclosed in glass pipettes and cooled at −10°C (freezing results in a slight coagulation of the tumor ascites rendering it easily sectionable). To the paraffin sections of such a coagulated ascites the acid hematein test was applied[1].

5) The acid hematein test was applied to sections of the carbowax-embedded solid tumor cut 18μ in thickness.

The results of the above three experiments were the same: that is to say, there

1) Baker's acid hematein test was initially applied to frozen sections, but Rennels (1953) found that this test could be applied equally well to paraffin-embedded material.

were found no BH-p type cells in any preparation.

If sectioning of the material would be responsible for the absence of BH-p type cells in the solid phase, the BH-p type cells should be observable in preparations with Experiment 3. If such cells are present only in the ascites, the BH-p type cells should be recognizable in preparations of Experiment 4. If the above two possibilities may exist there, the results derived from Experiment 5 would be understandable. It is then important to observe the tissue culture preparations based on the explants which have been taken from the tumor-bearing animal, not from the stock culture.

The application of the acid hematein test to the culture material of the methylcholanthrene-induced tumor tissue showed that BH-p type cells were found distributed in the peripheral zone of explants, as well as in the out-growth region. This finding is an indication that the BH-p type cells exist in the tumor tissue. In the inner zone of the explant, there is a difficulty in observing the cell reaction due to the thick layer of cells.

Further experiments were undertaken in order to learn the nature of the phospholipid-containing cells (BH-p type cells).

6) The sixth experiment deals with the treatment of the tumor ascites with low temperature. The MTK-sarcoma III was chosen as material. The experiment was carried out as follows:

a) Prepare nine glass pipettes for each run of the experiment. b) Collect samples of the tumor ascites in each pipette. On the other hand, an aliquot of the same ascites was fixed with formal-calcium and subjected to the test. c) Close both ends of each pipette by melting over the gas burner. d) Stock these pipettes in the refrigerator at about 0°C. e) Take out three pipettes each after 3, 6 and 10 days respectively: one is subjected to the acid hematein test and the other two are inoculated into the peritoneal cavity of two rats. The percentage of BH-p type cells in the ascites stored at low temperature decreased with days of storage: for instance, it was 18% in 3 day-stored ascites (Fig. 4), 0.5% in 6 day-stored ascites, and approximately 0% in 10 day-stored ascites. Incidentally, the percentages of the BH-p type cells in the original ascites was 40%.

On the other hand, the rats inoculated with the tumor ascites which was kept frozen at 0°C for 3 days showed without exception active tumor proliferation two days after inoculation, while in the rats inoculated with 6 day-stored tumor ascites such proliferation also occurred 13 to 15 days after inoculation. No tumor growth was found in rats which received the inoculation of 10 day-stored ascites.

The results presented above seem to indicate that the occurrence of many BH-p type cells in the ascites induce early tumor growth in rats.

In addition, the tumor ascites subjected to low-temperature treatment was

observed with the aid of a phase contrast microscope prior to implantation. Phase contrast microscopy studies made at 37°C have revealed that the treatment strikingly damages a majority of the cells: there remain only a small number of cells which are in a viable state (Figs. 2-3). These cells vary in size, and all show a high refractive index with the densely packed cytoplasm. Unfortunately, there is no positive evidence to show that these are tumor cells which take part in the tumor growth after inoculation into the peritoneal cavity of rats. It is then important to investigate the ability of these cells to promote tumor growth and to examine whether they are of the BH-p type or not. The following experiment was undertaken to answer the above questions, through the comparison between the living and fixed state of a single cell.

7) The methylcholanthrene-induced sarcoma cultured *in vitro* (MS 3) was chosen as meterial for this experiment. The culture material was exposed to low temperature.

Culture medium was discarded from the test tube in which two coverslips, each carrying four tumor explants, were inserted; then the tube was put into a vaccum flask containing ice and calcium chloride. The temperature in the flask ranged from −15° to −20°C. After 90 minute's freezing, the coverslips were taken out from the tube, mounted on a specially designed slide with a small amount of culture medium, and incubated at 37°C in a thermobox equipped with a phase contrast microscope.

The majority of the cells treated as above underwent degeneration; they showed a detachment of the cytoplasmic membrane from the cell body, presumably due to rapid hydration of the cytoplasm (Fig. 5). Besides these abnormal cells, there were to be seen a few cells, normal in appearance. The resident cells of normal appearance were generally spindle-like in shape and abundant in the zone immediately around the explant. Interestingly, these resident cells often showed an amoeboid movement during the course of phase microscopy examination for a period of several hours. A few of these cells were photographed and then coverslips were immersed in formal-calcium to subject the material to the acid hematein test. The results of the test showed that every resident cell possessed the phospholipid-containing cytoplasm; it is then evident that they are none other than BH-p type cells (Figs. 6-7). Generally the cytoplasm of damaged cells was negative to the test. Blue coloration was also shown by certain damaged cells. This indicates that none of them are BH-p type cells of resident type.

Discussion

Haven and Bloor (1956) have stated that the malignant tumor has been shown to possess a higher content of phospholipid and cholesterol than either benign

tumors or normal tissues of origin. In general, actively growing tissue such as the peripheral zone of a transplanted tumor was found to contain more phospholipid than the less active or even necrotic center (Bierich & Lang, 1933; Haven, 1937). Jowett (1931) ascertained by chemical analysis of human tissues, that growing cells, particularly in the case of malignant cells, have a high phosphatide-cholesterol ratio. He has expressed the view as follows.....phosphatides and cholesterol may play an important role in regulating protoplasmic structure and permeability of cell membrane. Although permeability to water-soluble substances in the resting cell may not be a limiting factor in maintenance of structure and metabolism, the growing cell, or more active resting cells, may require greater permeability to obtain its supplies. Hence, the growing cell, or resting cell of high metabolism, may show a high lecithin-cholesterol ratio.

This view may involve something about the nature of the BH-p type cells dealt with in the present paper. After the above views are taken into consideration the speculation is possible that the BH-p type cells may possess a high metabolic activity since they have a high phospholipid content.

As far as the present author is aware, there is little information available on the protoplasmic constituent of tumor cells having much higher lipid content than that of the normal cells. Albert and Johnson (1954) have measured the phospholipid phosphorus of both total and small particle fractions in normal liver and hepatoma by means of fractionation, and reported that there is no visible difference in phospholipid content per unit of nitrogen of the total and small particles between normal and tumor tissues.

On the basis of the results derived from Experiments 2, 3, 4 and 5 in this study it is probable that the phospholipid contained in BH-p type cells may be of exceedingly unstable nature, because the BH-p type cells could not be found in sections of the tumor, and the biochemical analysis made by other workers has not yet detected the increase of phospholipid in any cytoplasmic particulates.

In reference to the recorded evidence, the data presented in this study seem to indicate that the mitochondria of tumor cells contain less lipid than normal mitochondria, or that the tumor mitochondria contain the different type of lipid as compared to the normal ones, and that phospholipids of the BH-p type cells may exist in combination with cytoplasmic substances which are smaller in size than the mitochondria. Haven and Bloor (1956) suggested that a change in the type of lipid might occur during azo dye carcinogenesis.

The low temperature preservation of tumors over a long term has an obvious advantage as an alternative to the serial transplantation of tumors. Extensive work on the preservation of tumor in a frozen state has been done by Craigie (1954). The method employed in his experiments is different from that of the

present study, and therefore the comparison of the data is difficult between them. It seems, however, very reasonable to consider that "refractile cells" applied by Craigie to resident cells after refrigeration may correspond to "BH-p type cell" used by the present author, because of their morphological similarity.

The hypothesis of stem-cells as progenitors of the tumor was established by Makino with collaboration of his co-workers (Makino 1951; 1952 a, b; Makino & Kanô 1953, 1955; Makino & Tanaka 1953 a, b; Makino & Tonomura 1955) and by Hauschka and Levan (1953): the tumor cells most frequently occurring with characteristic chromosome-number together with a special ideogram form a stem-lineage. The distinct pattern of each tumor is associated with the genotype controlled by stem-cells.

It has been shown that the stem cells can survive under disadvantageous circumstances, for instance under drastic treatments, or cold storage. Probably they have a specific capacity to survive unfavorable conditions. The inquiry into the morphological and physiological nature of the resident cells (or probably the BH-p type cells) is of prime importance in cancer chemotherapy, since they are the primary source of tumor regrowth following subjection of the tumor cells to drastic treatments. In this connection the findings of the present study may not be of little value indicating that the resident cells may contain phospholipids in considerable amount in their cytoplasm.

Résumé

Morphological and cytochemical experiments have been undertaken in the present study with a hope to learn the nature of phospholipid-containing tumor cells (BH-p type cells). The results obtained indicate that: 1) phospholipids contained in BH-p type cells are of labile nature; 2) resident tumor cells following low-temperature exposure are characterized by phospholipid-rich cytoplasm; 3) with the increase in number of the BH-p type cells in the tumor ascites the tumor growth becomes speedy; and 4) there is a possibility that the BH-p type cells of resident type may correspond to tumor stem-cells which function as the principal progenitors of the tumor.

Acknowledgement: The writer wishes to express his deep appreciation to Professor Dr. Sajiro Makino for his keen interest in this study and for going through the manuscript.

Literature

Alberts, S. & R. M. Johnson 1954. Cancer Res. 14: 271-276.
Bierich, R. & A. Lang 1933. Z. Physiol. Chem. 216; 217-223.
Craigie, J. 1954. Adv. in Cancer Res., vol. 2, pp. 197-228.
Hauschka, T. S. and A. Levan 1953. Exp. Cell Res. 4: 457-467.

Hack, M. H. 1952. Anat. Rec. 112: 275-301. Haven, F. L 1937. Am. J. Cancer 29: 57-62.
Haven, F. L. & W. R. Bloor 1956. Adv. in Cancer Res. vol. 4, pp. 237-314.
Hori, S. H. 1957. Gann 48: Jowett, M. 1931. Biochem. J. 25: 1991-1998.
Makino, S. 1951. Proc. Jap. Acad. 27: 287-291.
———— 1952 a. Gann 43: 17-34.
———— 1952 b. Chromosoma 4: 649-674.
———— & K. Kanó 1953. J. Nat. Cancer Inst. 13: 1213-1235.
———— & ———— 1955. *Ibid.* 15: 1165-1181.
———— & T. Tanaka 1953 a. *Ibid.* 13: 1185-1199.
———— & ———— 1953 b. Gann 44: 39-46.
———— & A. Tonomura 1955. Z. Krebsforsch. 60: 597-608.
Rennels, E. G. 1953. Anat. Rec. 115: 659-670.

Explanation of Figures (Plate XXIV)

Fig. 1. A BH-p type cell found in the ascites stored for 6 days at 0°C. Acid hematein test. ×800.

Fig. 2 & 3. Surviving cells found in the ascites stored for 3 days at 0°C. Phase microscopy. ×1000.

Fig. 4. The 3 day-stored ascited at low temperature. Considerable numbers of BH-p type cells are observable. Acid hematein test. ×400.

Fig. 5. Cells damaged by rapid raise of temperature from −15°C to 37°C.

Fig. 6 & 7. Spindle-shaped cell sarcoma subjected to low temperature treatment. Coverslip culture. Fig. 6. fixed cells stained with acid hematein. Fig. 7, living cells, the same ones as those seen in Fig. 6.

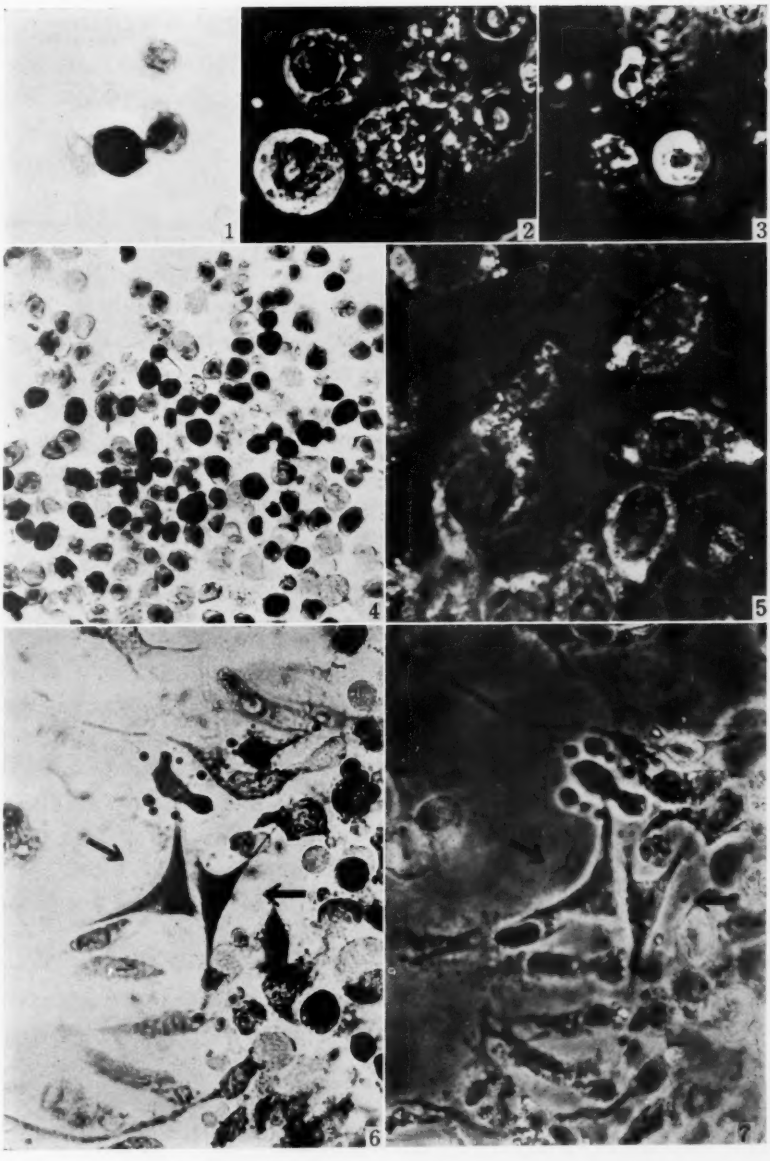
要　旨

腫瘍細胞の細胞化学的研究, III. 酸性ヘマテインに対して異った染色性を示す2種の腫瘍細胞について

堀　　浩 (北海道大学理学部動物学教室)

BH-p 型腫瘍細胞の本質を究明するために，形態学的ならびに細胞化学的研究を行ったところ，次の結果が得られた。

1) BH-p 型細胞に含まれている燐脂質は非常に不安定な形であって，しかもミトコンドリアより小さい細胞質内物質と比較的ゆるく結合しているのではないかと思われる。2) 腫瘍腹水を低温処理した際に生き残る残存腫瘍細胞は BH-p 型である。しかしながらすべての BH-p 型細胞が残存細胞になりうるとは限らない。3) BH-p 型細胞の数が多い程，腫瘍の成長は促進する。4) 生き残る BH-p 型細胞は，腫瘍の形成に第一義的にあづかる種族細胞に匹敵するものではないかと思われる。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[GANN, 48, 305~314; October, 1957]

STUDIES ON THE ORIGIN OF SERUM MUCOPROTEIN PRODUCTION—STUDIES PERTAINING TO INCREASED MUCOPROTEIN IN MALIGNANT TUMORS

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(Department of Internal Medicine & Cancer Institute, Sapporo Medical College)

A voluminous literature exists relating to increased serum mucoprotein (referred to as Mp) levels in cancer and other malignant tumors. Many problems, however, remain to be solved concerning the site of its production, its metabolism, and the differences between physiologic Mp and the increased Mp in malignant disorders.

Concerning the site of production of the pathologically increased Mp and glycoprotein, Seibert *et al.*[1,2,3] advocated a tissue destruction theory, whereas Shetlar *et al.*[4,5] postulated a tissue proliferation or repair theory. Winzler[6] compared the polarographic filtrate waves of the outflow blood of tumors with blood of the heart, and reported that the active substance causing the reaction originated in the tumor itself. Greenspan[7] divided the sites of production into hepatic and extrahepatic sources, owing to the fact that diminution of Mp was observed in cases with dysfunction of liver, such as cirrhosis of the liver; and its elevation was observed following the development of certain extrahepatic diseases, such as pneumonia, or hepatic tumor, either primary or metastatic. Furthermore, Gersh[8] and Catchpole[9,10] have suggested that the increase of serum Mp and glycoprotein may be due to the liberation of water-soluble substance from connective tissue by depolymerization of ground substances.

It is the purpose of the present studies to clarify the site of production of serum Mp and its metabolism, including both the physiologic Mp and the increased Mp in malignant tumors.

The Origin of Physiologic Mp and Its Metabolism

Materials and Methods: Wistar strain albino rats weighing approximately 180g and rabbits weighing approximately 2 kg were used for the experiments.

Serum Mp was determined as tyrosine content according to the method described by Winzler,[11] and was simultaneously determined as polarographic filtrate waves (PfWs), as introduced by Waldschmidt-Leitz.[12]

Moving-boundary electrophoresis (Tiselius method) of the sera was performed in both phosphate buffer (pH.=7.8, μ=0.14, 7.5V/cm, 40min.) and in acetate buffer

(pH.=4.5, μ=0.1, 6V/cm, 70min.) as reported by Mehl.[13]

In the experiment with normal rabbits, each 2 ml of blood was drawn from the femoral artery, femoral, hepatic, and portal veins, and the sera were used for comparison of the PfW. In the experiments with rats, 2 ml of blood was obtained from the jugular vein.

For the production of chronic liver injury, 40% CCl_4 solution dissolved in olive oil was administered orally to the rats at biweekly intervals, in doses of 2 ml per 180g body weight, for a period of 8 weeks. Two weeks after the last dose, PfW of the sera from these animals were compared to the corresponding PfW of the sera obtained prior to CCl_4 administration. Control animals received the equal doses of olive oil without CCl_4. At the conclusion of the experiment the animals were sacrificed and the livers were subjected to histological examination. Serum cobalt tests were performed as functional test of the liver in the group of rats given CCl_4.

Reticuloendothelial system (RES) blockade was performed as follows:

Blood samples were drawn prior to the test. Ten days thereafter, 0.5ml of silver colloid solutions, ranging from 1 to 3%, were injected intravenously per 180 g body weight, and the blood samples were obtained at 24 hours following the injection. Again polarographic studies were made on the pre-and postblockade sera. Adler-Reimann's congo red index and prothrombin times were run simultaneously in the experiment to ascertain the efficacy of RES blockade and effect of silver colloid on liver function.

Forssmann filtrate (Communin solution, obtained from the Fujisawa Co., Ltd.) was given to the rats to stimulate the RES. On eleventh day after the initial puncture, four groups of rats were injected subcutaneously with doses of 0.1, 0.2, 0.3, and 0.5ml of communin solution. Blood samples were drawn five hours after the injection. Control animals are the same as thosse of RES-blockade.

Results.

1. Comparison of the Mp content of pre- with posthepatic blood in normal rabbits:

Blood from the femoral artery and blood from the portal vein were defined as prehepatic blood, and blood from the hepatic vein as posthepatic blood (Fig. 1 and Fig. 2). No difference was found between femoral arterial and venous blood in tyrosine content and PfW.

Mp content in blood from the hepatic vein was, however, higher than in blood from the arterial blood. There was no significant difference between blood from the portal vein and hepatic vein in Mp content, perhaps due to the fact that the blood from the portal vein is greatly influenced by alimentary factors.

In summary, it appears that the Mp content of posthepatic blood is higher than

that of prehepatic blood, and from this fact we may postulate that physiologic Mp is liberated from the liver.

2. Results in rats with liver injury:

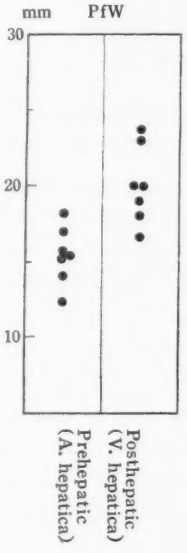

Fig. 1 Comparison of Pre and Posthepatic Sera. Significant at 5% level

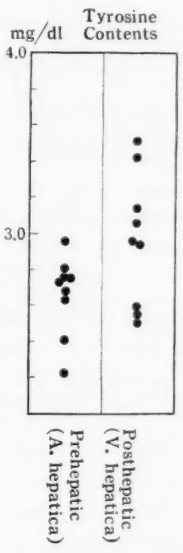

Fig. 2 Comparison of Pre and Posthepatic Sera. Significant at 5% level

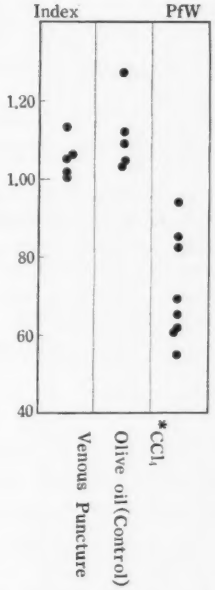

Fig. 3 Comparison of Untreated, Control, and CCl_4 treated Rats Sera.
Index: PfW of Post-treatment/PfW of Pre-treat.
*Significant at 5% level

Development of hepatic dysfunction in CCl_4 treated rats was ascertained from the findings of the functional tests and histological abnormalities, characterized by cellular degeneration and leukocyte infiltration in the area of central vein. PfW decreased significantly in these rats because of the decreased hepatic Mp production due to liver injury (Fig. 3).

3. Results of RES-blockade in rats:

Judging from congo red indices, a lowering of phagocytic function was observed in the 3% silver colloid treated rats, with no change in prothrombin time tests.

Electrophoretically, new peaks of M3 and M4 appeared together with M1 and M2 peaks, as noted by Mehl[14] (Fig. 4a).

PfW increased in proportion to the dose of silver colloid solution injected (Fig. 5a)

4. Results of RES stimulation in rats:

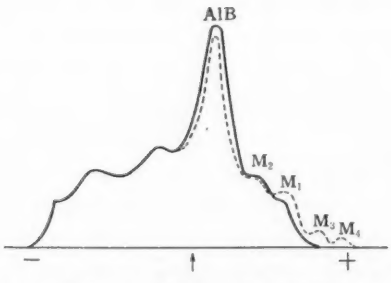

Fig. 4a Schema of Electrophoretic Pattern (Acetate Buffer) of Sera of Rats with Silver Colloid Injection.
Straight Line: Control Rats
Dotted Line: Rats injected with Silver Colloid Solution

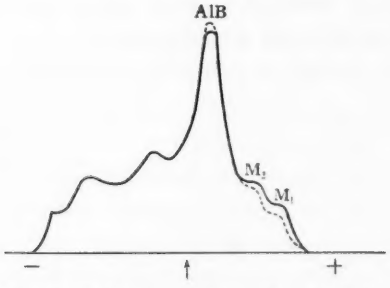

Fig. 4b Schema of Electrophoretic Pattern (Acetate Buffer) of Sera of Rats with Communin Injection.
Straight Line: Control Rats
Dotted Line: Rats injected with Communin Solution

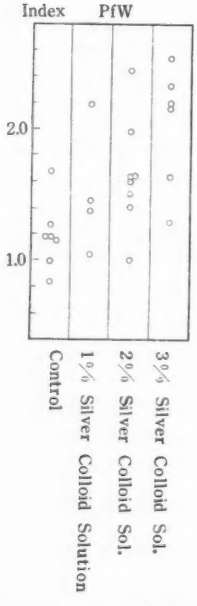

Fig. 5a Comparison of Sera of Control Rats with Rats injected with Various Concentration of Silver Colloid Sol.
Index: PfW of Post-treatment/PfW of Pre-treat.

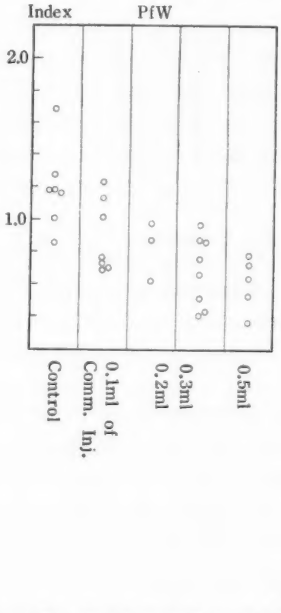

Fig. 5b Comparison of Sera of Control with Communin injected Rats.
Index: PfW of Post-treatment/PfW of Pre-treat.

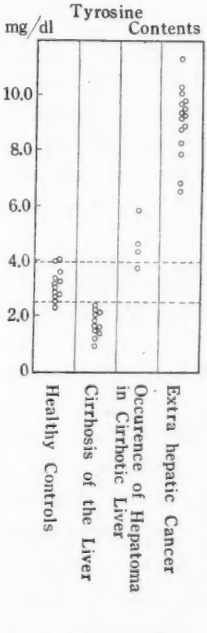

Fig. 6 Serum Mucoprotein Level

PfW decreased proportionately to the amount of Forssmann filtrate, communin, injected. Electrophoretically, M1 and M2 peaks decreased, and M3 and M4 peaks did not appear (Fig. 4b, Fig. 5b).

The Origin of Increased Mp Production and Its Metabolism in Malignant Tumors

Materials and Methods Clinically, in 11 cases of cirrhosis of the liver, 4 cases with hepatoma and 15 cases with extrahepatic cancer, serum Mp was determined as described above.

In the animal experiments, Wistar strain rats weighing approximately 180 g and adult fowls were used. The rats were previously bled and sera were separated for comparison with the post-treated sera.

Rats with experimentally induced chronic liver injury as described above were innoculated with ascitic fluid from rats with Yoshida-sarcoma. Sera of these animals were obtained 48 hours after inoculation.

Preparation of fowl sarcoma extract:

Three grams of fowl sarcoma tissue (Chiba-strain), 10ml of saline, and 100,000 units of penicillin solution were homogenized in a blender, centrifuged at 1,000 rpm for one minute, and the supernatant obtained. Approximately 2 ml of supernatant was injected subcutaneously between basilic and cephalic veins of the rooster's wing.

A localized tumor with tight adhesions to the underlying tissue was formed. Oxalated blood samples were obtained from the afferent (cephalic vein) and efferent vessels (brachial vein).

Serial administrations of RES blocking agent and tumor inoculum:

Twenty-four hours after silver colloid injection, the rats were inoculated with ascitic fluid of Yoshidasarcoma. Blood samples were obtained 48 hours after inoculation and compared to the specimens drawn prior to RES blockade.

Communin injections and tumor inoculation:

Rats were inoculated with ascitic fluid of Yoshida-sarcoma 5 hours after the initial communin injection. Thereafter, communin injections were repeated every 12 hours, and the subsequent blood sample was drawn 48 hours after Yoshida sarcoma inoculation. These sera and the sera of control animals were again subjected to similar determination.

Results.

1. Diminution of Mp level was observed clinically in cases with cirrhosis of the liver (Fig. 6), and the serum Mp level gradually increased following the occurence of hepatoma in a cirrhotic liver. The degree of increment was less than

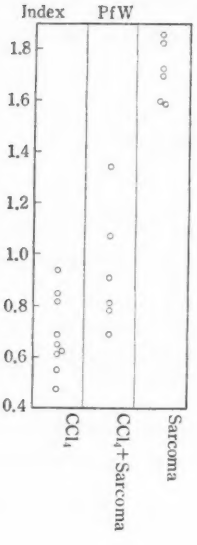

Fig. 7 Comparison of Sera of Rats with Liver injury, Liver injury plus Tumor and Tumor alone.
Index: PfW of Post-treatment / PfW of Pre-treat.

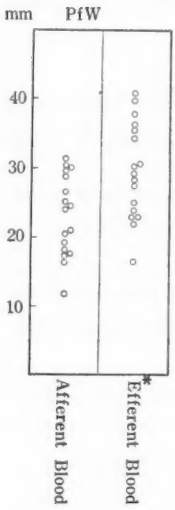

Fig. 8 Comparison of Afferent and Efferent Plasma (Fowl-Sarcoma)
* Significant at 1 % level

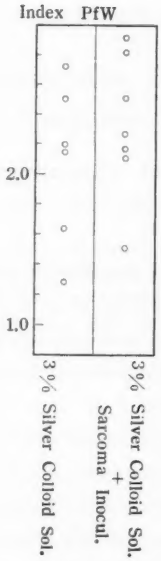

Fig. 9a Comparison of Sera of Silver Colloid injected Rats with Silver Colloid injected Rats with Sarcoma.
Index: PfW of Post-treatment / PfW of Pre-treat.

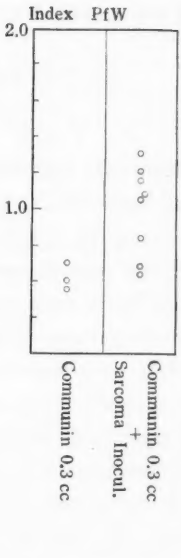

Fig. 9b Comparison of Sera of Communin injected Rats with Communin injected Rats with Sarcoma
Index: PfW of Post-treatment / PfW of pre-treat.

in the cases with extrahepatic cancer, in which the degree of liver injury was negligible.

2. Serum Mp levels were elevated following inoculation of Yoshida-sarcoma into CCl_4 treated rats. Comparing the serum Mp contents of CCl_4 treated rats to those that received CCl_4 treatment and sarcoma inoculations, the higher levels were obtained in the latter specimens, consistent with the clinical findings above (Fig. 7).

3. As shown in Table 1 and Fig. 8, Mp levels in efferent blood were significantly higher than in the afferent blood going to the tumor. Table 2 shows the electrophoretic patterns of plasma proteins obtained in this experiment. One albumin and 5 protein fractions were obtained with fowl plasma, as reported by Mckinley.[15] Among 5 protein fractions, the G_1 pear, as designated by the present authors,

Table 1 Comparison of Mucoprotein (Tyrosin) Content in Afferent and Efferent Plasma (Fowl Sarcoma)

	Before Sarcoma-Inoculation	After Sarcoma-Inoculation	
		Afferent Plasma	Efferent Plasma
1	1.85(mg/dl)	2.58(mg/dl)	2.60(mg/dl)
2	2.55	3.68	4.00
3	2.10	2.33	2.58
4	2.28	2.53	2.94
5	2.18	2.73	3.13
6	2.00	2.73	2.95
7	2.03	2.75	3.68
8	2.48	2.36	2.48
9	1.95	2.22	2.17
10	2.88	3.35	3.39
11	2.34	2.94	3.05

* Significant at 1% level

Table 2 Relative Distribution of Plasma Fractions of Fowl with Sarcoma Inoculation

	ALB	G_1	G_2	G_3	G_4	G_5
Afferent Plasma	33.6 ± 7.1	5.5 ± 1.4	8.8 ± 2.7	6.5 ± 0.9	11.8 ± 5.9	29.2 ± 5.7
Efferent Plasma	26.6 ± 7.1	10.3 ± 2.8	7.8 ± 1.3	7.8 ± 1.6	12.7 ± 6.2	28.8 ± 7.5

has coincided with the α_1-globulin fraction with respect to its mobility. Since the Mp fraction migrated with the α_1-globulin fraction, the resulting electrophoretic pattern has confirmed the result of Mp content and PfW determinations.

Further, it was interesting to note that the albumin fraction, contrary to the G_1 peak, was lower in efferent than in afferent blood. Since a diminution of the albumin fraction in malignant sera has been well known, it was likely that this phenomenon resulted from a consumption of albumin in the tumor tissue itself.

4. When an inoculation with Yoshida-sarcoma was given in the rats having induced RES-blockade by means of silver colloid solution (Fig. 9a), the PfW seemed to increase, as compared to the rats with RES blockade alone. Even in rats having increased RES function due to communin injection, following inoculation of sarcoma, there was a higher PfW than in rats with communin injection alone (Fig. 9b).

Discussion

Clinically, lowering of the PfW and diminution of the serum Mp content were noticed only in cases with cirrhosis of the liver. Study on the CCl_4 treated rats showed that the serum Mp level decreased in the presence of liver injury. Furthermore, Mp content of posthepatic blood was higher than that of prehepatic blood. Accordingly, it was supposed that the liver may be the source of physiologic Mp, as well as the other serum proteins.[16]

On the other hand, following RES blockade in rats with silver colloid solution (as ascertained by the congo red indices), the serum Mp level increased pro-

portionately to the amount of silver colloid administered. Conversely, the serum Mp level decreased proportionately to the amount of Forssmann filtrate, communin, administered.

Thus, it is likely that the effect of communin administration may not be due to any influence on antibody production or metabolism *per se*, but, rather, due to an acceleration of phagocytic function, since its effect appeared within 5 hours after subcutaneous injection. It is interesting to note that serum Mp levels increase with lowered phagocytic activity of the RES and, conversely, decrease with acceleration of its phagocytic activity. Accordingly, these results do not implicate Mp production, but, more probaly, a relationship between RES function and Mp metabolism.

When malignant tumor complicates preexistent liver injury (having low serum Mp levels prior to tumor development) a subsequent increase in serum Mp was noted both clinically and experimentally. In this respect, it is apparent that in the instance of using fowl sarcoma, the Mp is produced by the tumor tissue itself, and is accompanied by a simultaneous diminution of serum albumin. This is supported by Roberts[17] who reported that α-globulin increases with albumin consumption when tissue slice of liver, spleen, lymphnodes, and lymphosarcoma are incubated in sera.

Mp production, however, is not a simple consequence of albumin destruction, as shown by Winzler and Weimer,[18] who found a different amino acid composition of the two. Further, as pointed out by Catchpole *et al.* in their study of collagenase, Mp may have no direct relationship to serum albumin. The problem requires further investigation for elucidation of the relationships, if any, of these two compounds.

It is postulated that physiologic Mp is produced by parenchymatous cells of the liver, particularly those in the area of the central vein; whereas, the increased Mp in malignant tumor is liberated from tumor tissue itself. It is further possible that these two Mps may differ in their modes of metabolism.

Several interesting problems remain, such as whether or not the increased Mp in malignant tumors originates entirely in the tumor tissue and whether or not qualitative differences exist between physiologic Mp and the increased Mp of malignant tumor.

As shown in Fig. 7. the increase of Mp following sarcoma inoculation in rats with normal liver function is far greater than the increase of Mp following sarcoma inoculation in rats with liver injury. Also, clinically, the serum Mp gradually increases following development of hepatoma in a cirrhotic liver, while the increased Mp in cases of extrahepatic cancer without primary injury of the liver is far greater than when liver injury occurs. This may indicate that there exists an-

other Mp mobilized as a consequence of the malignant tumor but not produced by the tumor itself.

As for the biochemical significance of these Mps, it was found that the seromucoid isolated from sera of patients with cancer showed a higher catalase-depressing activity as compared to that of sera from healthy controls or patients with inflammatory disease. This phenomenon is to be the subject of a future report by the present authors.

Conclusion

Physiologic Mp is produced by the liver and is constantly present, while the specific Mp produced by malignant tumor tissue itself differs in its behavior with respect to RES phagocytic activity. It is suggested that this specific Mp may be the carrier of cancer toxin.

Summary

1) It was concluded that physiologic Mp is produced by the liver, from the fact that Mp content of posthepatic blood was higher than that of prehepatic blood, and serum Mp content decreased in the presence of both clinical and experimental liver damages.

2) Physiologic Mp level was assumed to be metabolized by the phagocytic function of RES, owing to the fact that Mp level increased with lowered phagocytic activity of the RES, and decreased with acceleration of its activity.

3) On the other hand, it was postulated that the increased serum Mp found in malignant tumor was liberated from the tumor tissue itself, by comparing the Mp content of afferent and efferent blood.

4) Further discussion was made concerning the existence of another source (perhaps in the liver) of Mp induced indirectly by the presence of the malignant tumor in addition to the Mp as produced by the tumor tissue, and that the Mp produced by the tumor tissue itself may be connected with the carrier of cancer toxin.

References

1) Seibert, F. B. et al.: J. Clin. Inv. **26**, 90 (1947).
2) Shedlovsky, T., Scudder, J.: J. Exp. Med. **75**, 119 (1942).
3) Weisbrod, F. G.: J. Lab. Clin. Med. **35**, 408 (1950).
4) Shetlar, M. R. et al.: Canc. Res. **9**, 515 (1949)., **10**, 681 (1950).
5) Keyser, J. W.: Brit. J. Canc. **8**, 238 (1954).
6) Winzler, R. J., Burk, D.: J. Nat. Canc. Inst. **4**, 417 (1944).
7) Greenspan, E. M., Trepper, B. et al.: J. Lab. Clin. Med. **39**, 44 (1952).
8) Gersh, I., Catchpole, H. R.: Am. J. Anat. **85**, 457 (1949).
9) Catchpole, H. R.: Proc. Soc. Exp. Biol. Med. **75**, 221 (1950).

10) Engel, M. B., Catchpole, H. R.: Proc. Soc. Exp. Biol. Med. **84**, 336 (1953).
11) Winzler, R. J., Devor, A. W., Mehl, J. W., Smyth, I. M.: J. Clin. Inv. **27**, 609 (1948).
12) Waldschmidt-Leitz, E., Mayer, K.: Physiol. Chem. **261**, 2 (1939).
13) Mehl, J. W., Humphrey, J., Winzler, R. J.: Proc. Soc. Exp. Biol. Med. **72**, 106 (1949).
14) Mehl, J. W., Golden, F., Winzler, R. J.: Proc. Soc. Exp. Biol. Med. **72**, 110 (1949).
15) Mc Kinley, W. P., et al.: Proc. Soc. Exp. Med. **84**, 346 (1953).
16) Miller, L. L., Bale, W. F.: J. Exp. Med. **99**, 125 (1954).
Canc. Res. **14**, 582 (1954).
18) Weimer, H. E., Mehl, J. W., Winzler, R. J.: J. Biol. Chem. **185**, 561 (1950).

要　旨

血清ムコ蛋白の産生起源
とくに悪性腫瘍の際増量するムコ蛋白について

和田武雄，大原弘通，佐々木忠男，中島二郎，谷内　昭
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癌および悪性腫瘍の際血清ムコ蛋白が著明に増量するが，これと生理的に存在するムコ蛋白との異同について研究している。

生理的に存在するムコ蛋白は，実験的並びに臨床的に肝機能障害時に低下し，また肝前および肝後血清の比較から肝内産生が結論された。また網内系機能低下時血中に遊離し，亢進時には補捉されるかの如く血中減少が起り，その動的平衡は網内系機能と密接に関係する。

悪性腫瘍の際増量するムコ蛋白は，臨床的にも肝外産生を推論せしめるが，腫瘍の輸出入血液のムコ蛋白比較実験から，腫瘍内産生ムコ蛋白の存在が確認された。一方悪性腫瘍の際増量するムコ蛋白の一部には，生体反応（防禦反応）的な意義の下に恐らくは肝で産生されるものの存在も推論され，われわれは腫瘍内産生ムコ蛋白を特異的 MP，反応性増量ムコ蛋白を非特異的 MP と名付けた。悪性腫瘍（癌）患者血清並びに尿から単離したムコ蛋白は非癌および正常のそれに比し著明にカタラーゼ抑制作用を現わすことから，腫瘍内産生ムコ蛋白は悪液質毒（癌腫毒）の担体としての意義を有するのではないかと考えている。さらに生理的ムコ蛋白と異なり，網内系により代謝され難い事実が推論された。

Book Review

THREE NEW FASCICLES OF THE ATLAS OF TUMOR PATHOLOGY

Three important facicles were recently added to the compiled edition of the Atlas of Tumor Pathology series, which has been in publication through the last ten years by the U. S. Armed Forces Institute of Pathology under the auspices of the committees of the National Academy of Science. As the over dozen fascicles already reviewed in this column, they are extremely well prepared and documented for comprehensive classifications and clear illustrations of the important lesions in the particular organs. They have also the common feature of excellent presentation of clinicopathological relationships of each tumor and are recommedable to all pathologists and surgeons interested in the human oncology as the first and best reference.

Section X, Fascicle 36: Tumors of the Pituitary Gland and Infundibulum, by Drs. Kernohan, J. W., and Sayre, G. P., 81 pp., 67 figg., & 3 tabb., published in 1956, $ 100—Using the materials from a series of 5,000 intracranial tumors treated at the Mayo Clinic, Drs. Kernohan and Sayre discuss in detail the pituitary neoplasms and hypopituitarism not associated with primary tumor of the pituitary gland. Among the formers, their descriptions are pretty well focused upon the chromophobe and eosinophilic adenomas. The illustrations are excellent, including two nice color plates. Clinicopathological comments are particularly well documented.

Section X, Fascicle 38: Tumors of the Eye and Adnexa, by Dr. Reese, A. B., 205 pp., 122 figg., and 15 tabb., published in 1956, $ 2.00—Dr. Reese covers the entire field of oncology of the orbit with many excellent illustrations, one in color plate, and tables in a fascicle much handier than his famous synonymous monograph. Neoplasms of rare incidence, extraocular tumors of epithelial, glial and mesodermal origins, and also the pseudotumors are discussed. Because pathologists of most of the countries are not regularly well accustomed with ophthalmologic pathology, this new contribution of Dr. Reese will find a real welcome by them. The very reasonable cost of the fascicle is also attractive.

Section VIII, Fascicle 30: Tumors of the Kidney, Renal Pelvis and Ureter, by Drs. Lucke, B., and Schlumberger, H. G., 208 pp., 187 figg., and 9 tabb., published in 1957, $ 2.25—Drs. Lucke and Schlumberger handle the great complexity of the renal and ureteral neoplasms in a compact leaflet of 200 pages with a brilliant

clarity. Their arrangement of the materials in a short general remark, heteroplastic tissues, benign mature tumors both of parenchymal and mesenchymal origins, and immature parenchymatous tumors, is very comprehensible. Greater parts are devoted to the renal adenocarcinoma, especially in connection with their clinicopathological behaviors. In the sections of the pelvis and ureter, the focuses are upon the metaplasia and neoplasia of the epithelial elements. An embryogenetic review of the tumors is omitted, perhaps with purpose. Illustrations, both gross and microscopic, leave nothing to be desired.

The above fascicles are available at the American Registry of Pathology, Armed Forces Institute of Pathology, Washington 25, D. C., United States of America.

(Kunio Oota)

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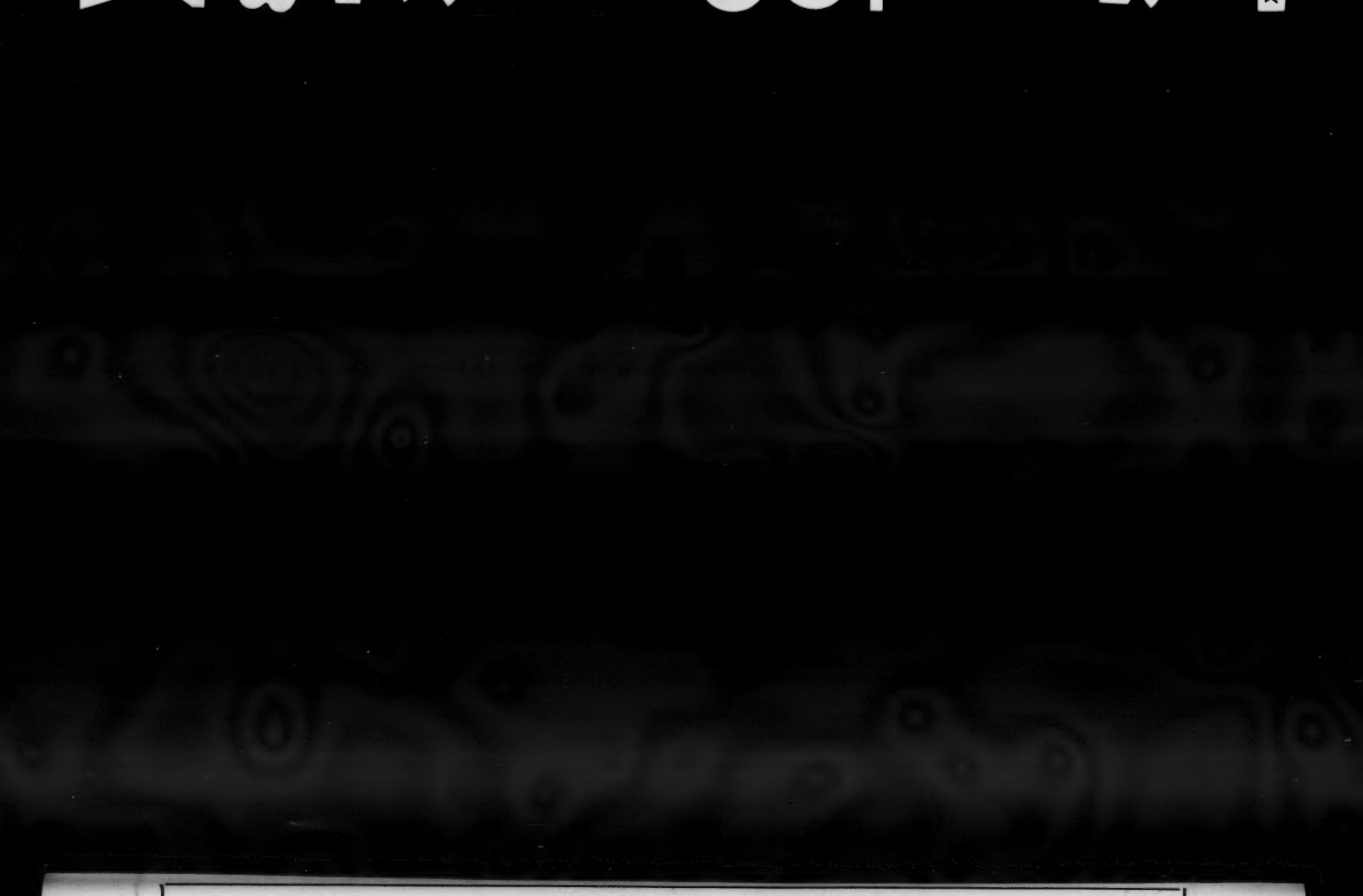
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